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(71) 出願人 (米国を除く全ての指定国について): 麒麟麦酒株式会社 (KIRIN BEER KABUSHIKI KAISHA) [JP/JP]; 〒104-8288 東京都中央区新川二丁目10番1号 Tokyo (JP). 工業技術院長が代表する日本国 (JAPAN as represented by SECRETARY OF AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY) [JP/JP]; 〒100-8921 東京都千代田区霞が関一丁目3番1号 Tokyo (JP).

(72) 発明者; および
(75) 発明者/出願人 (米国についてのみ): 千葉靖典 (CHIBA, Yasunori) [JP/JP]. 貝沼真美 (KAINUMA, Mami) [JP/JP]. 竹内 誠 (TAKEUCHI, Makoto) [JP/JP]. 川島永子 (KAWASHIMA, Eiko) [JP/JP]. 吉田 聡 (YOSHIDA, Satoshi) [JP/JP]. 山野重幸 (YAMANO, Shigeyuki) [JP/JP]; 〒236-0004 神奈川県横浜市金沢区福浦1-13-5 麒麟麦酒株式会社 基盤技術研究所内 Kanagawa (JP). 地神芳文 (JIGAMI, Yoshifumi) [JP/JP]; 〒300-1233 茨城県牛久市栄町6-255 Ibaraki (JP). 石井智子 (ISHII, Tomoko) [JP/JP]; 〒305-0042 茨城県つくば市下広岡1055-588 Ibaraki (JP). 新間陽一 (SHIMMA, Yoh-ichi) [JP/JP]; 〒305-0031 茨城県つくば市吾妻1-408-301 Ibaraki (JP).

(74) 代理人: 平木祐輔, 外 (HIRAKI, Yusuke et al.); 〒105-0001 東京都港区虎ノ門一丁目17番1号 虎ノ門5森ビル3階 Tokyo (JP).

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(57) Abstract: Novel yeast variants capable of producing a glycoprotein wherein a sugar chain having the same sugar chain structure as a sugar chain produced by mammalian cells is attached to an asparagine residue of a protein; and a process for producing the sugar chain and the glycoprotein by a sugar chain engineering technique with the use of these variants. By using the auxotrophic triplex or tetraplex variant newly bred, a neutral sugar chain identical with the high-mannose type produced by mammalian cells (human cells, etc.) or a glycoprotein having the same neutral sugar chain can be efficiently produced at a high purity. By transferring a mammalian type sugar chain biosynthesis-associated gene into such a variant, it is also possible to efficiently produce a mammalian sugar chain of high-mannose type, hybrid type, complex type, etc. or a protein having a mammalian type sugar chain.

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NOVEL YEAST VARIANTS AND PROCESS FOR PRODUCING GLYCOPROTEIN CONTAINING MAMMALIAN TYPE SUGAR CHAIN

Inventors and
Inventors/Applicants (only for US): Yasunori Chiba [JP/JP], Mami Kainuma [JP/JP], Makoto Takeuchi [JP/JP], Eiko Kawashima [JP/JP], Satoshi Yoshida [JP/JP] and Shigeyuki Yamano [JP/JP]
Kirin Beer K.K.
Basic Research Center,

1-13-5 Fukuura, Kanazawa-ku,
Yokohama-shi, Kanagawa-ken;
Yoshifumi Jigami [JP/JP],
6-255 Sakae-cho,
Ushihisa-shi,
Ibaraki-ken;
Tomoko Ishii [JP/JP],
1055-588 Shimohirooka,
Tsukuba-shi, Ibaraki-ken;
Yoh-ichi Shimma [JP/JP],
1-408-301 Azuma,
Tsukuba-shi, Ibaraki-ken

Applicant (for all designated states except US):

Kirin Beer K.K. [JP/JP],
2-10-1 Shinkawa, Chuo-ku,
Tokyo-to
Japan as represented by Secretary of Agency of Industrial Science and Technology [JP/JP]
1-3-1 Kasumigaseki
Chiyoda-ku, Tokyo-to

Agent:

Yusuke Hiraki et al.
1-17-1 Toranomon, Toranomon 5-ban Bldg., 3rd Floor,
Minato-ku, Tokyo-to

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Field of the technology

This invention pertains to novel yeast variants having an ability to produce a glycoprotein with a saccharide chain having the same saccharide chain structure as that of a saccharide chain produced by a mammalian cell added at the asparagine residue of a protein, and a process for

producing the saccharide chain and glycoprotein using the variants and a sugar chain engineering technique.

Prior art

There are two kinds of natural proteins, that is, simple proteins comprising amino acids alone and complex proteins having saccharide chains, lipids or phosphate bound to them, and most cytokines have been known to be glycoproteins. Some of them such as erythropoetin (EPO), tissue plasminogen (TPA), etc., have been found to lose their original biological activities if their saccharide chains are removed [Yo Kibata, Tanpakushitu, Kakusan, Koso, 36:775-788 (1991)]. The saccharide chains are predicted to have an essential role for the expression of a biological activity, but the correlation between saccharide chain structure and biological activities is not necessarily clear, and it is necessary to develop technology to allow freely changing and controlling the structure of the saccharide chains (kind of saccharide, bonding site, chain length, etc.) to be added to proteins.

The saccharide chains of glycoproteins can be classified Asn-binding type, mucin type, O-GlcNAc type, GPI anchor type, proteoglycan type, etc. [Makoto Takeuchi, "Glycobiology Series 5, Glycotechnology," Yo Kibata, Sen-ichiro Hakomori and Katutaka Nagai, eds., Kodansha Scientific, pp. 191-208 (1994)]. They have respectively specific biosynthetic pathways and play respective physiological roles. The biosynthetic pathway of the Asn-bonding saccharide chain has been studied well and analyzed in detail.

In the biosynthesis of an Asn-bonding saccharide chain, a precursor comprising N-acetylglucosamine, mannose and glucose is synthesized on a lipid carrier intermediate, and it is first transferred to a glycoprotein-specific sequence (Asn-X-Ser or Thr) in the endoplasmic reticulum (ER). Subsequently, it is processed (cleavage of a glucose residue and a specific mannose residue), forming an M8 high mannose saccharide chain ($Man_8GlcNAc_2$) comprising 8 mannose residues and 2 N-acetylglucosamine residues. The protein containing this high mannose saccharide chain is transported to the Golgi body where it is modified in various ways, and this modification inside the Golgi body is markedly different between yeast and mammals [Gemmill, T.T. and R.B. Trimble, Biochim. Biophys. Acta. 1426:227 (1999)].

In the case of mammalian cells, several mannose residues are cleaved in many cases as a result of α-mannosidase I acting on a high mannose saccharide chain. The saccharide chain ($Man_8GlcNAc_2$) formed in this process is a saccharide chain called a high mannose saccharide chain. When N-acetylglucosaminyl transferase (GnT) I is allowed to act on a M5 high mannose saccharide chain ($Man_5GlcNAc_2$) with 3 mannose residues cleaved, 1 N-acetylglucosamine residue is transferred, forming a saccharide chain comprising $GlcNAcMan_5GlcNAc_2$. The

saccharide chain prepared using these procedures is called a hybrid saccharide chain. Furthermore, if α-mannosidase II and GnT-II are allowed to act, a saccharide chain structure called complex, that is, $GlcNAc_2Man_3GlcNAc_2$ is formed, and furthermore, if a group of some ten kinds of saccharide transferases are allowed to act to add N-acetylglucosamine, galactose, sialic acid, etc., various mammalian saccharide chains are formed (Figure 1). In mammals, all of these high mannose, hybrid and complex saccharide chains are found, but the kind of saccharide chains bonded to them depends on the proteins, and even for a single protein, different kinds of saccharide chains may be bonded. These saccharide chains, depending on their types and other saccharide chains bonded, exhibit superior functions in glycoprotein biosyntheses, intracellular sorting, shielding antigens, biostability, organ targeting characteristics, etc., [Tamao Endoh, "Tousakougaku" [Saccharide chain engineering], Sangyo Shosakai, pp. 64-72 (1992)].

In the case of erythropoietin, which is the first glycoprotein drug in history produced using a gene-recombinant animal cell as a host cell, the importance of the saccharide chain has been pointed out. The saccharide chain of erythropoietin inhibits binding to the receptors, but it has exhibited convincing improvement in retaining the active structure and behavior inside the body and is essential for expression of the pharmacological activity as a whole [Takeuchi and Kobata, Glycobiology 1:337-346 (1991)]. Furthermore, there is a strong correlation between the pharmacological effects of erythropoietin and its structure, the kind and the number of branches (number of branches formed by GlcNAc bonded to $Man_3GlcNAc_2$) of its saccharide chain [Takeuchi, et al., Proc. Natl. Acad. Sci., USA 86:7819-7822 (1989)] . In an erythropoietin having an undeveloped branch structure, renal clearance is hastened, as a result retention time inside the body is shortened, and this phenomenon is reported to be the main cause of the behavior [Misaizu, et al., Blood 86:4097-4104 (1995)]. There is a similar example observed in the case of serum glycoproteins such as fetuin, etc., and it has been found that if the terminal sialic acid of the saccharide chain is removed to expose galactose, the glycoprotein is recognized by lectins on the hepatic cell surface, causing it to disappear quickly from the bloodstream [Ashwell and Harford, Annu. Rev. Biochem. 51:531-554 (1982); Morell, et al., J. Biol. Chem. 243:155-159 (1968)].

Furthermore, a phosphate group is added to a mannose residue at the 6^{th} position at the nonreducing terminal of the high mannose saccharide chain by many of a group of enzymes localized in human lysosomes after biosynthesis and being transported to the Golgi body, forming a lysosome enzyme-specific recognition marker. Furthermore, through bonding with a high-affinity receptor, the mannose-6-phosphate receptor (MPR), they are distinguished from other proteins, transported to the prelysosome, and after dissociation from the MPR under acidic conditions, they are transported further to the lysosome [von Figura and Hasilik, Annu. Rev.

Biochem. 54:167-193 (1984)]. This lysosome enzyme-specific phosphate group addition reaction is carried out with two kinds of enzymatic reactions, and if there is any genetic defect in their genes, it is known to generate abnormalities in the mechanism for targeting the lysosome, causing a critical morbid state generically called lysosome disease [Leroy and DeMars, Science 157:804-806 (1967)]. Therefore, although one can say, mammalian saccharide chains in short, their functions depend markedly on their structures.

On the other hand in yeasts, mannan saccharide chains (external saccharide chains) are formed having several mannose residues to 100 residues or more added to M8 high mannose saccharide chains. The biosynthesis of external saccharide chains in *Saccharomyces* is considered to proceed in the pathways shown in Figures 2 and 3* [Ballou, et al., Proc. Natl. Acad. Sci., USA 87:3368-3372 (1990)]. Specifically, the reaction to start extending the chain is carried out first by adding mannose to an M8 high mannose saccharide chain through α-1,6 bonding (Figure 2, reaction I, B). The enzyme involved in carrying out this reaction has been found to be a protein coded by the OCH1 gene [Nakayama, et al., EMBO J. 11:2511-2519 (1992)]. Furthermore, the reaction consecutively extending mannose with α-1,6 bonding (Figure 2, II) is carried out forming poly α-1, 6 bonds [sic] mannose bonds, which becomes the skeleton of the external saccharide chain (Figure 2, E). The mannose bound by α-1,6-bonds has α-1,2-bonds at mannose branches (Figure 2, 3: c, F, H), and at the end of this branched mannose with α-1,2-bonds, the addition of mannose by an α-1,3-bond may be carried out (Figure 2, 3: D, G, H, I). This α-1,3-bonded mannose addition is attributable to the MNN1 gene product [Nakanishi-Shindo, et al., J. Biol. Chem. 268:26338-26345 (1993)]. Furthermore, it has been found that an acidic saccharide chain with mannose-1-phosphate added to the high mannose saccharide chain portion and an external saccharide chain portion is also formed [Figure 2, * ; sites of possible phosphorylation corresponding I-labeled sites in formula (I)]. This reaction has been found to be attributable to a gene [sic] coded by the MNN6 gene [Wang, et al., J. Biol. Chem. 272:18117-18124 (1997)], and furthermore, the gene (MNN4) coding for the protein positively controlling this rearrangement reaction has also been elucidated [Odani, et al., Glycobiology 6:805-810 (1996); Odani, et al., FEBS Letters 420:186-190 (1997)].

In many cases, the external saccharide chain produces a heterogenous protein product making protein purification difficult or reducing the relative activity [Bekkers, et al., Biochim. Biophys. Acta 1089:345-351 (1991)]. Furthermore, because of markedly different saccharide chain structures, the same biological activities as those of mammalian-origin glycoproteins are not detected in glycoproteins produced by yeast, and they are strongly immunogenic in

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* [Translator's note: No figures found in the copy provided. It is difficult to translate the following several sentences without these figures.

mammals. Therefore, yeasts have been considered to be unsuitable hosts for the production of useful mammalian-origin glycoproteins. The development of a yeast strain which makes possible the production of glycoproteins having the same biological activities as those of mammalian-origin glycoproteins, that is, glycoproteins containing mammalian saccharide chains has been a hope in the scientific community and in industry.

Therefore, to allow yeast to produce mammalian saccharide chains, it becomes essential to isolate a variant having a saccharide chain synthetic system wherein the reactions described above causing addition of many molecules of mannose, which is a modifier of the saccharide chains of yeast specific glycoproteins, are not carried out, no external saccharide chain is added, and the saccharide chain synthesis is terminated at the stage of a M8 high mannose saccharide chain. Subsequently, it is necessary to insert the gene allowing mammalian saccharide chain biosynthesis from the M8 high mannose saccharide chain, which is a precursor of the mammalian saccharide chain, into this yeast variant.

To obtain a glycoprotein lacking an external saccharide chain, the use of strains lacking a group of enzymes for producing the external saccharide chains of yeasts has been studied. To obtain a defective strain, there are methods of obtaining a variant by genetic mutation using a chemical, UV irradiation or natural mutations, and methods which artificially destroy the target gene.

There are many reports to date on the former methods. For example, the mnn2 variant has a defect in a branching step, forming α-1,2 bonds from the α-1,6 skeleton of the external saccharide chain, and the mnn1 variant has a defect in a step forming α-1,3 bonded mannose at the branched end. However, these variants do not have any defect in the α-1,6-mannose bonding comprising the skeleton of the external saccharide chain; they respectively form an external saccharide chain with a long chain length. Furthermore, the mnn7, -8, -9 and -10 variants have been isolated as a variant having 4-15 molecules of α-1,6 bonded mannose, but in these variants only their external saccharide chains are shortened, and the saccharide chain propagation of high mannose saccharide chains is not stopped [Ballou, et al., J. Biol. Chem. 255:5986-5991 (1980); Ballou, et al., J. Biol. Chem. 264:11857-11864 (1989)]. Addition defects in the external saccharide chains are also observed, for example in secretion variants such as sec18, where the protein transport from the endoplasmic reticulum to the Golgi body is temperature-sensitive. However, in sec variants, the secretion of the proteins itself is inhibited at a high temperature, and consequently it is not suitable for the production of secreted glycoproteins.

Therefore, these variants are considered unsuitable as host yeast cell for producing mammalian saccharide chains because no complete biosynthesis of the desired high mannose saccharides chains is possible.

On the other hand, the saccharide chain biosynthetic pathway in the endoplasmic reticulum (ER) has been elucidated by isolating variants with defects in various biosynthetic stages and analyzing them biochemically. In the case of the alg (asparagine-linked glycosylation) variants, a unique method of introducing [^{3}H]-mannose was used for selection because the variant has less intake than the wild strain cells, avoiding any damage or killing by the radiation, and being concentrated. Among these, the alg3 variant shows accumulation of Dol-pp-$GlcNAc_2$-Man_5 (Dol-pp is dolichol pyrohosphate) at a nonpermissive temperature [Tanner, W., et al., Biochim. Biophys. Acta. 906:8199 (1987)]. Furthermore, Chikami, et al. carried out analyses using the Δochlmnnlalg3 triple variant [Chikami, et al., Tanpakushitsu Kakusan Kohso 39 (4):657 (1994)]. If the mannan protein saccharide chain was analyzed after fluorescent labeling with PA (2-aminopyridine), two peaks coinciding with Man_8lcNAc_2-PA and $Man_5GlcNAc_2$-PA were observed. The former among them was found to be identical to the ER core saccharide chain from the results of analyses of the α-1,2-mannosidase digestion reaction, FAB-MS, etc. On the other hand, the latter yielded $Man_2GlcNAc_2$-PA as a result of α-1,2-mannosidase digestion and removal of 2 molecules of Man as a result, and as a result of a treatment (partial acetolysis), specific cleavage of α-1,6 bonded Man, 1 molecule of Man was removed. From the results obtained, the saccharide chain of $Man_5GlcNAc_2$-PA produced by the triple variant was found to have the incomplete core saccharide chain structure shown in the formula (II). Incidentally, as a reason why triple variant forms not only $Man_5GlcNAc_2$ but also $Man_8GlcNAc_2$, the alg3 mutation accumulation of $Man_5GlcNAc_2$-pp-Dol on dolichol pyrophosphate is leaky.

On the other hand, as a result of advances in genetic engineering techniques in recent years, it has become possible to construct defective strains with several target genes destroyed.

Some yeast species have been known to have auxotrophs, and as a specific example of such an auxotroph, there are the leu2, trp1, ura3, ade2 and his3 mutations [Taiji Ohshima, ed., "Seibutukagaku Jikkenhou 39, Kobo Bunshiidengaku Jikkenhou" [Handbook for biochemical experiments 39, yeast molecular genetics experimental methods], pp. 119-144 (1996)]. If the original unmutated gene is inserted, the auxotrophism can be cancelled, and proliferation is possible without adding the essential component to the culture medium. Based on this principle, it is possible to carry out gene destruction in yeasts (Figure 4). In this method, the target gene DNA on the plasmid is cleaved or partially deactivated by in vitro procedures, and a suitable selection marker gene DNA is inserted there, forming a structure having the selection marker sandwiched between the upstream side and downstream side of the target gene. Subsequently, linear DNA having this structure is introduced into the yeast cell, inducing a double recombinant between the two ends of the fragment inserted and the corresponding portions of the target gene on the chromosome, substituting a DNA structure with the selection marker inserted [Rothstein,

Methods Enzymol. 10:202-211 (1983)]. In this method, one selection marker is required to destroy one gene.

Japanese Kokai Patent Application Nos. Hei 6[1994]-277086 and Hei 9[1997]-266792 disclose molecular breeding of yeast strains lacking external saccharide chains. However, the glycoprotein saccharide chain produced by the double variant (Δoch1 Δmnn1) disclosed in Japanese Kokai Patent Application No. Hei 6[1994]-277086 was found to contain an acidic saccharide chain having a phosphate residue. This acidic saccharide chain structure is a structure not found in saccharide chains of mammalian origin such as humans, it is recognized as a foreign substance inside the mammalian body and is antigenic [Ballou, Methods Enzymol. 185:440-470 (1990)]. Therefore, it was decided to construct a quadruple variant (disclosed in Japanese Kokai Patent Application No. Hei 9[1997]-266792) by further destroying the functions of the gene (MNN4) positively controlling mannose-1-phosphorate transferase and mannose transferase enzyme gene (KRE2) to carry out the reaction extending an O-bonded saccharide chain. The glycoprotein saccharide chain produced by the yeast strain described in this patent publication was found to have the desired M8 high mannose saccharide chain. Furthermore, a strain with the *Aspergillus saitoi*-origin α-1,2-mannosidase gene inserted in the above gene-destroyed yeast was found to have a high mannose saccharide chain ($Man_{5-8}GlcNAc_2$) with 1 to several mannose residues cleaved [Chiba, et al., J. Biol. Chem. 273:26298-26304 (1998)].

Furthermore, Shimma, et al. prepared another kind of quadruple variant by inserting alg 3 [Shimma, Y., et al., Mol. Gen. Genet. 256:469-480 (1997); Wang, et al., J. Biol. Chem. 272:18117-18124 (1997); Yoichi Shimma and Yoshifumi Chikami, 32nd Yeast Genetics Forum, Abstracts, p. 64 (1999); Shimma, Y., et al., Abstracts of XIX International Conference on Yeast Genetics and Molecular Biology, p. 443 (1999)].

The saccharide chains of glycoproteins produced by these yeast strains are also found in mammals and show no antigenicity, but as is apparent from the example of erythropoietin, these high mannose saccharide chain-containing glycoproteins are predicted not to show activities equivalent to those of glycoproteins prepared from mammalian cells because of the saccharide chain structure. Furthermore, in this quadruple variant, the subsequent use of the 4 selective markers (leu2, ura3, lys2 and trp1), recessive genetic mutations of the host, becomes impossible. One of the remaining auxotrophic markers is destroyed artificially instead of by mutation, and consequently the recombination on the yeast chromosome utilizing the homology of these marker gene sites on the chromosome cannot be carried out. Consequently, the introduction of multiple genes is difficult for saccharide chain hydrolase genes, saccharide transferase genes and glyconucleotide transporter genes necessary for producing mammalian saccharide chains as well as genes for carrying out the production of useful glycoproteins. As described above, it is known

that there are more than ten saccharide transferases required for the production of mammalian saccharide chains, and consequently the use of this yeast cell as a host is considered to be unsuitable for optional modification and control of the saccharide chain structure.

The objective of this invention is to overcome the problems described above in the production of Asn-binding glycoproteins in yeast and provides a method for the production of saccharide chains having the same saccharide chain structures as those of the high mannose, hybrid and complex saccharide chains produced by human cells and other mammalian cells and glycoproteins containing these saccharide chains, using yeast.

Presentation of the invention

The inventors of this invention studied diligently, and they found that it was possible to produce saccharide chains having the same saccharide chain structures as those of mammalian saccharide chains using a novel yeast variant (auxotrophic triple variant) with the gene (OCH1) coding for α-1,6-mannosyl transferase to carry out the initial chain propagation addition reaction, the gene (MNN1) coding for α-1,3-mannosyl transferase adding mannose to the non-reducing terminal of the saccharide chain, and the gene (MNN4) controlling the addition of mannose-1-phosphorate destroyed with the auxotrophic mutation maintained as a selection marker, that is, without finally inserting genes complementing auxotrophy, and furthermore that it was possible to produce various mammalian saccharide chains by inserting the mammalian saccharide chain biosynthesis gene into the variant.

Specifically, this invention pertains to the following items (1)-(14).

(1) A yeast variant having a glycoprotein-producing ability characterized by having the phenotype of the och1, mnn1 and mnn4 mutations and at least 4 or more kinds of auxotrophic mutant strain, as well as the ability to produce a glycoprotein containing an oligosaccharide chain represented by the following formula (I) as an asparagine-binding saccharide chain.

//Insert the formula (I) pg 8//

(In the formula, Man shows mannose, and GlcNAc shows N-acetylglucosamine. * shows a possible phosphorylation site.)

(2) A yeast variant having a glycoprotein-producing ability characterized by having the phenotype of the och1 mutation with the *OCH1* gene destroyed (Δoch1), the mnn1 mutation with the MNN1 gene destroyed (Δmnn1) and the mnn4 mutation with the MNN4 gene destroyed (Δmnn4) without finally inserting genes complementing auxotrophy and at least 1 or more kind of auxotrophic mutant strain as well as the ability to produce a glycoprotein containing an oligosaccharide chain represented by the above-mentioned formula (I) as an asparagine-binding saccharide chain.

(3) The yeast variant of Claim 1 or 2, wherein the auxotrophic phenotype is selected from the ura3, his3, leu2, ade2, trp1 and can1 mutations.

(4) The yeast variant of Claim 3, wherein the yeast belongs to the family *Saccharomyces*.

(5) The yeast variant of Claim 4, wherein the yeast belongs to *Saccharomyces cerevisae*.

(6) The yeast variant of Claim 5, wherein the yeast is *Saccharomyces cerevisae* TIY19.

(7) A process for the production of an oligosaccharide chain characterized by culturing one of the yeast variants of Claims 1-6 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain represented by the above-mentioned formula (I) as an asparagine-binding saccharide chain to form and accumulate in the culture, collecting the glycoprotein from the culture and recovering the oligosaccharide from the glycoprotein collected.

(8) A process for the production of a glycoprotein characterized by culturing one of the yeast variants of Claims 1-6 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain represented by the above-mentioned formula (I) as an asparagine-binding saccharide chain to form and accumulate in the culture and collecting the glycoprotein from the culture.

(9) A process for the production of a glycoprotein characterized by culturing one of the yeast variants of Claims 1-6 transformed by a recombinant plasmid containing the gene coding for a mammalian-origin asparagine-binding glycoprotein in a culture medium, allowing the glycoprotein containing an oligosaccharide chain represented by the above-mentioned formula (I) as an asparagine-binding saccharide chain to form and accumulate in the culture and collecting the glycoprotein from the culture.

(10) A yeast variant characterized by inserting at least two or more mammalian saccharide chain biosynthesis genes into a yeast variant having the och1, mnn1 and mnn4 phenotype.

(11) A yeast variant characterized by inserting at least one or more mammalian saccharide chain biosynthesis genes into a yeast variant of Claims 1-6.

(12) A process for the production of an oligosaccharide chain characterized by culturing the yeast variants of Claim 10 or 11 in a culture medium, allowing a glycoprotein containing an

oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture, collecting the glycoprotein from the culture and recovering the oligosaccharide from the glycoprotein collected.

(13) A process for the production of a glycoprotein characterized by culturing the yeast variants of Claim 10 or 11 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture and collecting the glycoprotein from the culture.

(14) A process for the production of a glycoprotein characterized by culturing the yeast variants of Claim 10 or 11 transformed with a recombinant plasmid containing the gene coding for a mammalian-origin asparagine-binding glycoprotein in a culture medium, allowing the glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture and collecting the glycoprotein from the culture.

The inventors of this invention also found that various mammalian saccharide chains could be produced without inserting the α-mannosidase II gene, which is a gene related to mammalian saccharide chain biosynthesis, but introducing other mammalian saccharide chain biosynthesis genes using a novel yeast variant (auxotrophic quadruple variant) prepared by allowing the above yeast variant (auxotrophic triple variant) with the gene (OCH1) coding for α-1,6-mannosyl transferase to carry out the initial chain propagation addition reaction, the gene (MNN1) coding for α-1,3-mannosyl transferase to add mannose to the non-reducing terminal of the saccharide chain, and the gene (MNN4) controlling the addition of mannose-1-phosphorate to be destroyed, and in addition the gene (ALG3) related to saccharide biosyntheses in the ER to be destroyed.

Specifically, this invention pertains to the following items (15)-(30).

(15) A yeast variant having a glycoprotein-producing ability characterized by having the phenotype of the och1, mnn1, mnn4 and alg3 mutations and at least 5 or more kinds of auxotrophic mutant strains as well as the ability to produce a glycoprotein containing an oligosaccharide chain represented by the following formula (II) as an asparagine-binding saccharide chain.

//Insert the formula (II) pg 11//

(In the formula, Man shows mannose, and GlcNAc shows N-acetylglucosamine.)

(16) A yeast variant having a glycoprotein-producing ability characterized by having the phenotype of the och1 mutation with the OCH1 gene destroyed (Δoch1), the mnn1 mutation with the MNN1 gene destroyed (Δmnn1), the mnn4 mutation with the MNN41 gene destroyed (Δmnn4) and the alg3 mutation with the ALG3 gene destroyed (Δalg3) without finally inserting genes complementing auxotrophy and at least 1 or more kind of auxotrophic mutant strain as well as the ability to produce a glycoprotein containing an oligosaccharide chain represented by the above-mentioned formula (II) as an asparagine-binding saccharide chain.

(17) The yeast variant of Claim 15 or 16, wherein the auxotrophic phenotype is selected from the ura3, his3, leu2, ade2, trp1 and can1 mutations.

(18) The yeast variant of Claim 17, wherein the yeast belongs to the family *Saccharomyces*.

(19) The yeast variant of Claim 18, wherein the yeast belongs to *Saccharomyces cerevisae*.

(20) The yeast variant of Claim 19, wherein the yeast is *Saccharomyces cerevisae* YS134-4A.

(21) A process for the production of an oligosaccharide chain characterized by culturing one of the yeast variants of Claims 15-20 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain represented by the above-mentioned formula (II) as an asparagine-binding saccharide chain to form and accumulate in the culture, collecting the glycoprotein from the culture and recovering the oligosaccharide from the glycoprotein collected.

(22) A process for the production of a glycoprotein characterized by culturing one of the yeast variants of Claims 15-20 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain represented by the above-mentioned formula (II) as an asparagine-binding saccharide chain to form and accumulate in the culture and collecting the glycoprotein from the culture.

(23) A process for the production of a glycoprotein characterized by culturing one of the yeast variants of Claims 15-20 transformed by a recombinant plasmid containing the gene coding for a mammalian-origin asparagine-binding glycoprotein in a culture medium, allowing the glycoprotein containing an oligosaccharide chain represented by the above-mentioned formula (II) as an asparagine-binding saccharide chain to form and accumulate in the culture and collecting the glycoprotein from the culture.

(24) A yeast variant characterized by inserting at least two or more mammalian saccharide chain biosynthesis genes into a yeast variant having the och1, mnn1, mnn4 and alg3 phenotype.

(25) A yeast variant characterized by inserting at least one or more mammalian saccharide chain biosynthesis genes into a yeast variant of Claims 15-20.

(26) A process for the production of an oligosaccharide chain characterized by culturing the yeast variants of Claim 24 or 25 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture, collecting the glycoprotein from the culture and recovering the oligosaccharide from the glycoprotein collected.

(27) A process for the production of a glycoprotein characterized by culturing the yeast variants of Claim 24 or 25 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture and collecting the glycoprotein from the culture.

(28) A process for the production of a glycoprotein characterized by culturing the yeast variants of Claim 24 or 25 transformed with a recombinant plasmid containing the gene coding for a mammalian-origin asparagine-binding glycoprotein in a culture medium, allowing the glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture and collecting the glycoprotein from the culture.

(29) A yeast strain having an α-mannosidase II activity characterized by having the α-mannosidase II gene inserted.

(30) A process for the production of α-mannosidase II characterized by culturing the yeast strain of Claim 29 in a culture medium and collecting the α-mannosidase II formed and accumulated in the culture.

This specification includes the details described in the specification and/or drawings of Japanese Patent Application No. Hei 11[1999]-233215, which is the basis of the priority claims of this patent application.

Brief description of the figures

Figure 1 a drawing showing the biosynthetic pathways of conventional N-bonded saccharide chains.

Figure 2 is a drawing showing the biosynthetic pathways of the N-bonded saccharide chains of yeast (*S. cerevisiae*). In the figure, H, C and E proceed to I, D and F, respectively, of Figure 3.

Figure 3 is a drawing showing the biosynthetic pathways (continuation) of N-bonded saccharide chains of yeast (*S. cerevisiae*).

Figure 4 shows the previous method for destroying yeast genes.

Figure 5 shows a method for destroying genes without finally inserting the genes complementing auxotrophy.

Figure 6 shows the results of structural analyses of the TIY19 strain cell surface layer mannan protein saccharide chain.

Figure 7 shows the results of structural analyses of TIY19 strain cell surface layer mannan protein saccharide chain with the α-1,2-mannosidase gene inserted using an Amide-80 column.

a: mannan saccharide protein saccharide chain of the TIY19 strain

b: mannan saccharide protein saccharide chain of the TIY19 strain with the α-1,2-mannosidase gene inserted

Figure 8 shows the results of structural analyses of TIY19 strain cell surface layer mannan protein saccharide chain with the α-1,2-mannosidase gene inserted using an ODS-80™ column.

a: standard saccharide chain having the structure shown by formula (III)

b: fraction obtained in Figure 6

Figure 9 shows the results of GnT-I activity measurements.

Figure 10 shows the results of structural analyses of the TIY19 strain cell surface layer mannan protein saccharide chain with the α-1,2-mannosidase and GnT-I genes inserted using an Amide-80 column.

A: saccharide chain structural analysis of the TIY19 strain with the vector alone inserted

B: saccharide chain structural analysis of the TIY19 strain with the α-1,2-mannosidase and GnT-I genes inserted

a: $Man_5GlcNAc_2$-PA

b: $GlcNAcMan_5GlcNAc_2$-PA

c: $Man_6GlcNAc_2$-PA

d: $Man_7GlcNAc_2$-PA

e: $Man_8GlcNAc_2$-PA

Figure 11 shows the results of structural analyses of the TIY19 strain cell surface layer mannan protein saccharide chain with the α-1,2-mannosidase and GnT-I genes inserted using an ODS-80^{TM} column.

A: mixture of standard products

B: fraction collected in Figure 10, B

Figure 12 shows the results of Western blot analyses using the cell extract of the YPH500 strain with the α-mannosidase II gene inserted.

A: results of Western blot analyses of the cell extract of the YPH500 strain with only the vector (pYEX-BX-3HA) inserted

B: results of Western blot analyses of the cell extract of the YPH500 strain with the chimeric α-mannosidase II gene-containing vector (pYEOM2-HA) inserted

Figure 13 shows the results of α-mannosidase II activity measurements carried out using the cell extract of the YPH500 strain with the α-mannosidase II gene inserted.

A: results of activity measurements on the cell extract of the YPH500 strain with only the vector (pYEX-BX-3HA) inserted

B: results of activity measurements on the cell extract of the YPH500 strain with chimeric α-mannosidase II gene-containing vector (pYEOM2-HA) inserted

a: $GlcNAcMan_5GlcNAc_2$-PA

b: $GlcNAcMan_8GlcNAc_2$-PA

Figure 14 shows the results of structural analyses of the FGF saccharide chains of the TIY48 strain with the FGF gene inserted (upper row) and the TIY53 strain with the FGF and α-1,2mannosidase genes inserted (lower row), using an Amido-80 column.

Explanation of symbols

GlcNAc, GN: N-acetylglucosamine

Man, M: mannose

PA: 2-aminopyridyl derivative

Brief explanation of the sequence tables

Sequence No. 1 shows primer A for amplifying the 5' region of the MNN1 gene.

Sequence No. 2 shows primer B for amplifying the 5' region of the MNN1 gene.

Sequence No. 3 shows primer C for amplifying the 3' region of the MNN1 gene.

Sequence No. 4 shows primer D for amplifying the 3' region of the MNN1 gene.

Sequence No. 5 shows primer E for amplifying the 3' region of the MNN4 gene.

Sequence No. 6 shows primer F for amplifying the 3' region of the MNN4 gene.

Sequence No. 7 shows primer G for amplifying the 5' region of the MNN4 gene.

Sequence No. 8 shows primer H for amplifying the 5' region of the MNN4 gene.

Sequence No. 9 shows primer I for amplifying the 5' region of the ALG3 gene.

Sequence No. 10 shows primer J for amplifying the 5' region of the ALG3 gene.

Sequence No. 11 shows primer K for amplifying the 3' region of the ALG3 gene.

Sequence No. 12 shows primer L for amplifying the 3' region of the ALG3 gene.

Sequence No. 13 shows primer M for amplifying the N terminal region of the α-mannosidase II gene.

Sequence No. 14 shows primer N for amplifying the N terminal region of the α-mannosidase II gene.

Sequence No. 15 shows primer O for amplifying the central region of the α-mannosidase II gene.

Sequence No. 16 shows primer P for amplifying the central region of the α-mannosidase II gene.

Sequence No. 17 shows primer Q for amplifying the C terminal region of the α-mannosidase II gene.

Sequence No. 18 shows primer R for amplifying the C terminal region of the α-mannosidase II gene.

Sequence No. 19 shows sequence S of double-strand DNA coding for the gene allowing the HA-tag to be repeatedly bound 3 times.

Sequence No. 20 shows sequence T of double-strand DNA coding for the membrane piercing region of the OCH1 gene.

Sequence No. 21 shows primer U for amplifying a portion of the catalytic region of the α-mannosidase II gene.

Sequence No. 22 shows primer V for amplifying a portion of the catalytic region of the α-mannosidase II gene.

Sequence No. 23 shows primer W for amplifying the human UDP-GlcNAc Transporter gene.

Sequence No. 24 shows primer X for amplifying the human UDP-GlcNAc Transporter gene.

Sequence No. 25 shows primer Y for amplifying the human prepro α-factor FGF gene.

Sequence No. 26 shows primer Z for amplifying the human prepro α-factor FGF gene.

Best embodiment of the present invention

This invention is explained in detail as follows.

The phenotypes required for the yeast variants of this invention are mutant varieties of the external saccharide chain biosynthesis genes specific to yeast, specifically, the och1, mnn1 and mnn4 mutations or the och1, mnn1, mnn4 and alg3 mutations.

Namely, they may be natural or artificial mutant strains as long as they are these mutant varieties.

Furthermore, the auxotrophic phenotypes for inserting foreign alien genes into the yeast variants of this invention are specified depending on the yeast strain used, and they are specifically selected from the ura3, his3, leu2, ade2, trp1 and can1 mutations. The number of auxotrophic phenotypes depends on the number of genes to be inserted, but generally one auxotrophic phenotype is required for one inserted gene. In the case of the insertion of multiple genes, the longer the gene fragment to be inserted, the lower the insertion efficiency, thus the lower the expression efficiency, and consequently, the larger the number of genes to be inserted, the more auxotrophic phenotypes required.

The "genes complementing auxotrophy" in this invention are genes for biosynthetic systems of biological components such as amino acids, nucleic acids, etc. The phenotype includes mutations causing these genes not to function, and thus the complementing genes are the genes with the original functions themselves. Therefore, the original yeast strain-origin genes are desirable.

Furthermore, the phrase "without finally inserting the genes complementing auxotrophy" means that one or more selection markers, that is, the auxotrophic phenotypes are utilized to destroy one or more genes (insert phenotype), after destruction, the corresponding phenotypes remaining are the same number as the number of genes destroyed, and the same corresponding phenotypes may be repeatedly usable in the case of another gene destruction (see Figure 5).

The yeast variants of this invention retaining auxotrophic phenotype for inserting foreign genes and having the gene for biosynthesis of an external saccharide chain specific to yeast destroyed (called auxotrophic variants, below) can be prepared as follows.

First of all, in the case of isolation of the DNA gene fragments necessary for destruction of a target gene, it is possible to obtain a gene fragment containing the target gene region from a public institution such as the US ATCC (American Type Culture Collection) (ATCC Recombinant DNA materials, 3rd edition, 1993) because the conformation on the chromosome has been elucidated (Goffeau et al., Nature, 387 (suppl.), 1-105 (1997)) as a result of the genomic project for *Saccharomyces cerevisiae*. Furthermore, the genomic DNA of *S. cerevisiae* can be extracted using conventional procedures, and the desired gene can be selected. This

extraction of the genomic DNA of *S. cerevisiae* can be carried out according to, for example, the method of Cryer, et al. [Method in Cell Biology 12:39-44 (9)] and method of Philippsen, et al. [Methods Enzymol. 194:169-182 (1991)].

The target gene is destroyed after carrying out amplification with the PCR method. The PCR method enables in vitro amplification of a specific fragment some 100 thousand times or more in about 2-3 h by combining a sense-antisense primer at the two ends of the region of the fragment, a heat-resistant DNA polymerase, a DNA amplification system, etc., and, for target gene amplification, a 25-30mer synthetic single-strand DNA is used as a primer, and the genomic DNA is used as a template.

The destruction of the target gene in this invention can be carried out by basically following the method disclosed in Rothtein, Methods Enzymol. 101:202-211 (1983). In this method, the target gene DNA on a plasmid is cleaved or partially deactivated, a suitable selection marker gene DNA is inserted at the site in order to prepare a structure with the selection marker sandwiched between the upstream side and downstream side of the target gene, and subsequently this prepared structure is inserted into the yeast cell. By carrying out the above procedures, double recombination is carried between the two ends of the fragment introduced (DNA structure having the selection marker inserted) and the corresponding portions of the target gene on the chromosome are substituted by the target gene on the chromosome with the fragment inserted.

Specifically, this is explained using the preparation of a MNN1 gene-destroyed strain as an example. A hisG-URA3-hisG cassette is cleaved with restriction enzymes from a plasmid constructed by Alani, et al., having a salmonella hisG gene DNA fragment bound to both ends of the URA3 gene [Alani, et al., Genetics 116:541-545 (1987)], inserted into the target gene on a plasmid constructed with a destroyed allelic gene. The target gene on the chromosome is substituted with this prepared plasmid in order to obtain a gene-destroyed strain. The URA3 gene inserted into the chromosome is between two strands of hisG, and homologous recombination between the hisG sequences causes 1 copy of hisG to drop out of the chromosome. The target gene on the chromosome still has 1 copy of the hisG fragment left, which is left as destroyed, but the host cell takes on Ura expression (Figure 5). This homologous recombination between hisG can be carried out with 5-fluoroorotic acid (5-FOA). The ura3 variant is resistant to 5-FOA [Boeke, et al., Mol. Gen. Genet. 197:345-346 (1984); Boeke, et al., Methods Enzymol. 154:165-174 (1987)], and the cell strain expressing Ura3 cannot grow in a 5-FOA medium. Therefore, if a resistant strain is isolated in a medium with 5-FOA added, reusing the procedures for URA3 is possible.

The same procedures are used in the MNN1 gene-destroyed strain to carry out MNN4 and OCH1 gene destruction in order to obtain the desired auxotrophic triple variant (Δoch1Δmnn1Δmnn4) of this invention. Furthermore, the same procedures are reused to carry out ALG3 gene destruction in order to obtain the auxotrophic quadruple variant (Δoch1Δmnn1Δmnn4Δalg3) of this invention.

Therefore, in the "artificially destroyed strain," in order to carry out artificial gene destruction using the above method, the auxotrophic phenotypes of the original yeast strain is not damaged by the gene destruction procedures. Consequently, the number of auxotrophic phenotypes of the artificial variant is equal to the number of auxotrophic phenotypes of the original yeast strain whether it is a triple or quadruple variant, which means at least one or more.

On the other hand, in the "natural mutant" with natural gene destruction instead of the above artificial technique, the above method is not used, and there is no relationship to the increase or decrease of the number of auxotrophic phenotypes.

If a yeast strain having 6 auxotrophic phenotypes is used for destruction of the OCH1, MNN1 and MNN4 genes in the case of a yeast variant producing the M8 high mannose saccharide chain of this invention, only 3 auxotrophic phenotypes are left, and the number of auxotrophic phenotypes of the variant becomes 4 or more.

Furthermore, if a M8 high mannose saccharide chain-producing yeast variant has the ALG3 gene mutation in addition to mutations in the OCH1, MNN1 and MNN4 genes, the natural variant of the mnn1 and alg3 mutations can be used in the case of preparation by the prior method, but the further destruction of the OCH1 and MNN4 genes is necessary, and consequently two auxotrophic phenotypes are used. Therefore, if the above yeast strain having 6 auxotrophic phenotypes is used, 4 auxotrophic phenotypes are left, and the number of auxotrophic phenotypes of the variant prepared is at least 5 or more.

Incidentally, the selection marker usable in the above procedures is not necessarily limited to auxotrophic markers such as URA3, etc., but those markers giving resistance against drugs such as G418, cerulenin, aureobasidin, zeosin, canavanine, cycloheximide, tetracycline, etc., are also usable. Furthermore, gene insertion and destruction can be carried out using gene markers providing resistance against solvents such as methanol, ethanol, etc., resistance to osmotic pressure due to glycerol, salts, etc., and metal ions such as copper ions, etc.

The insertion of DNA into a cell and transformation by the above procedures can be carried out using a conventional method, for example, if a phage is used as a vector, the method allowing an *E. coli* host to be infected with it may be used, allowing the host to take up the DNA efficiently. Furthermore, as a yeast transformation method using a plasmid, there is a method for treatment with a potassium salt for ready uptake of DNA before carrying out plasmid insertion or

electrical methods of inserting DNA into the cell [Becker and Guarente, Methods Enzymol. 194:182-187 (1991)].

Furthermore, the isolation, purification, etc., of DNA in the above procedures can be carried out using conventional methods, for example, in the case of *E. coli*, DNA extraction can be carried out using the alkali/SDS method and ethanol precipitation, and furthermore the DNA purification can be carried out with RNase treatment, PEG precipitation, etc. In addition, the determination of the DNA sequence of the genes, etc., can be carried out using conventional methods, for example, the dideoxy method (Sanger, et al., Proc. Natl. Acad. Sci., USA 74:5436-5467 (1977)], etc. Furthermore, the above DNA base sequence determination can be carried out easily using a commercially available sequencing kit, etc.

The auxotrophic variants prepared as described above can produce high mannose mammalian saccharide chains, and in order to allow them to produce hybrid and complex mammalian saccharide chains, saccharide chain hydrolase and transferase genes specific to yeast are inserted into the variants. Furthermore, the biosynthesis of the saccharide chains is originally carried out on the ER and in the Golgi body as discussed above, and the glyconucleotide starting materials of the saccharide chains must be present in these apparatuses, but there is no transporter for these glyconucleotides in yeast, or even if there is it is only a trace amount in the apparatuses where the saccharide chains are actually produced. Therefore, glyconucleotide transporter genes are necessary so that the glyconucleotides biosynthesized in the cytoplasm can be transported to the ER and Golgi body.

Therefore, the genes described above for saccharide chain hydrolases, transferases and glyconucleotide transporters are called "genes for the mammalian saccharide chain biosynthesis system" in this invention.

As a saccharide chain hydrolase gene, there are genes for -mannosidase (-mannosidase I, -mannosidase II, etc.), as a saccharide transferase gene, there are genes for N-acetylglucosaminyl transferise (GnT-I, GnT-II, GnT-III, GnT-IV, GnT-V), galactosyl transferise (GalT), fucosyl transferase (FucT), etc., and as a glyconucleotide transport gene, there are genes for UDP-GlcNAc transporter, UDP-Gal transporter, etc. These genes can be mammalian-origin isolated natural genes or synthetic genes.

The "mammalian saccharide chain biosynthesis genes" described above are those genes belonging to one, two or more kinds of the groups of genes described above, and they are used in the number required to produce the desired saccharide chain. In the case of multiple gene insertion, these genes may belong to the same or different groups.

The above auxotrophic variants or variants with a group of foreign genes inserted in the auxotrophic variants are cultured in a culture medium, as a result, the content of external saccharide chains specific to yeast is reduced, and those glycoproteins containing Asn-bonding saccharide chains identical to high mannose saccharide chain ($Man_{5-8}GlcNAc_2$), hybrid saccharide chain ($GlcNAcMan_5GlcNAc_2$) and complex saccharide chain ($Gal_2GlcNAc_2Man_3GlcNAc_2$) produced by mammalian cells are produced inside or outside the yeast cells.

Specifically, if the triple variant (*och1 mnn1 mnn4*) is used as an auxotrophic variant, the -mannosidase I and GnT-I gene 3 are inserted into the variant, allowing it to produce a hybrid saccharide chain. Furthermore, if the mammalian saccharide chain biosynthesis genes (-mannosidase II, GnT-II, GalT, UDP-GlcNAc transporter and UDP-Gal transporter genes) are inserted, a double chain composite saccharide chain ($Gal_2GlcNAc_2Man_2GlcNAc_2$) can be produced.

Furthermore, by inserting the GnT-IV and GnT-V genes, it is possible to allow the yeast to produce triple and quadruple chain composite saccharide chain.

Furthermore, if the quadruple variant (*och1 mnn1 mnn4 alg3*) is used as an auxotrophic variant, a double chain composite saccharide chain ($Gal_2GlcNAc_2Man_2GlcNAc_2$) can be prepared by inserting the mammalian saccharide chain biosynthesis genes (-mannosidase I, GnT-I, GnT-II, GalT, UDP-GlcNAc transporter and UDP-Gal transporter genes) without inserting the -mannosidase II gene.

To prepare saccharide chains and glycoproteins in high yields the above enzymes are desirably expressed in high concentrations in appropriate apparatuses (such as the Golgi body). Therefore, it is effective to use genes which match the codon usage frequency of yeast. Furthermore, to allow specific enzymes to be localized in specific apparatuses, it is also effective to add the signal sequences, etc., of yeast. Gene insertion can be carried out using various vectors such as the 2 μm plasmid type (YEp type), the chromosome-incorporation type (YIp type), etc., which are selected depending on the purpose. The YEp type vector enables multiple gene copy insertion and thus large quantity expression of the gene inserted. On the other hand, the YIp type vector allows the gene to be present in the chromosome, making stable retention possible. The promoters required for gene expression are structural expression promoters such as GAPDH, PGK, etc., induction expression promoters such as GAL1, CUP1, etc., and their use is not especially restricted but, for the production of saccharide chains, the use of these structural expression promoters is desirable. However, in the case of one or multiple expression of hydrolases, saccharide transferases and glyconucleotide transporter genes, the proliferation of the

yeast may be affected, and in such a case it is necessary to consider the use of induction promoters or the order of gene insertion.

Furthermore, the auxotrophic variants of this invention include, in addition to the above variants prepared by artificially destroying genes, those variants obtained using chemicals, ultraviolet irradiation or as a result of natural mutation. By inserting the above mammalian saccharide chain biosynthesis genes (hydrolase, transferase and glyconucleotide genes), this natural variant is allowed to produce mammalian saccharide chains and glycoproteins containing mammalian saccharide chains.

In addition, in order to allow the yeast to produce a glycoprotein of a different biological origin having the above saccharide chain, a gene is prepared by ligating the gene (cDNA, etc.), coding for the desired glycoprotein downstream of a promoter capable of expression in yeast is prepared, homologous recombination is carried out to incorporate it in the yeast host described above, or the above host is transformed by inserting into it the plasmid in order to obtain a transformant of the above host, which is cultured using known procedures, and the desired glycoprotein produced inside or outside the yeast cell is recovered.

The culture of the above yeast variants can be carried out using any of the methods conventionally used for culture of yeast. For example, it is possible to use a synthetic culture medium (containing nitrogen sources, carbon sources, inorganic salts, amino acids, vitamins, etc.), prepared by adding various kinds of culture medium components available from Difco but excluding any amino acids that can be supplied by the markers necessary for duplication and maintenance of the plasmid [Sherman, Methods Enzymol. 194: 3-57 (1991)].

To isolate and purify the glycoprotein from the culture (culture solution and cultured biomass), any of the conventional protein isolation and purification methods is usable.

For example, after completing the culturing, the cells grown are recovered by centrifugation, suspended in an aqueous buffer solution, and pulverized using an ultrasonic pulverizer, French press, Manton-Gaulin homogenizer, Dynomill, etc. to obtain a cell-free extract solution. From a supernatant prepared by centrifugation of the cell-free extract solution, the glycoprotein can be isolated and purified using conventional protein isolation and purification methods such as solvent extraction, salting out using ammonium sulfate, etc., anion-exchange chromatography using a resin such as diethylaminoethyl(DEAE)-Sepharose, etc., cation exchange chromatography using a resin such as S-Sepharose FF (Pharmacia), hydrophobic chromatography using a resin such as butyl cellulose, phenyl cellulose, etc., gel filtration using molecular sieves, affinity chromatography using His·Bind resins (Novagen), etc., chromatofocusing method, electrophoresis such as isoelectric point electrophoresis, etc., alone or in combination.

Application examples

This invention is explained specifically in detail using application examples as follows. However, these examples do not restrict the technical range of this invention at all.

Application Example 1

Breeding of the yeast variant (mnn1 mnn4 och1 auxotrophic triple variant) having an ability producing mammalian saccharide chains

(1) Preparation of the mnn1 auxotrophic variant and properties

A cassette (HUH) having the salmonella hisG gene ligated in a direct repeat manner on both sides of the URA3 gene was excised from pNK51 as already reported [Alani, et al., Genetics, 116: 541-545 (1987)] using BglII and BamHI and inserted into an *E. coli* plasmid pSP73 at the BamHI site. The plasmid prepared was named pSP73-HUH.

The MNN1 gene is located near the 5th chromosomal centromere of yeast, and the DNA base sequence of the MNN1 gene is registered in the GenBank database as L23753 [Yip, et al., Proc. Natl. Acad. Sci., USA 9: 2723-2727 (1994)]. The 5' region of the MNN1 gene was amplified using PCR with primer A (GGATCCGAAGAAAACCTAATACATTGAAGT: sequence No. 1) and primer B (GCATGCCCTTTGGTTTAATATAAATCTCCGGAGTGC: sequence No. 2), and the 3' region was amplified with primer C (GCATGCTACATAACTCCAATCAGCAGCAAATATGTC: sequence No. 3) and primer D (GCGGCCGCGTGTTCTGTTCGGGTAACGTTTAAACCAAT: sequence No. 4). These DNA fragments were incorporated into plasmid pHYH having a HIS3 marker at the SphI site in order to prepare pHYH mnn1. To destroy the MNN1 gene with the HUH cassette, a SphI fragment of 1.8 kb was obtained from pHYH mnn1and inserted at the SphI site of pSP73-HUH to construct pSP73-Δ-mnn1::HUH. The plasmid was cleared at the NotI site to obtain a straight chain, and a wild strain W303-1A (MATa leu2-3, 112his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100) was transformed using the lithium acetate method [Ito, et al., J. Bacteriol. 153: 163-168 (1983)]. After transformation, it was sown on SD-Ura culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases except uracil and an amino acid mixture (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant.

The genomic DNA of the transformant was prepared, and the PCR method was used to confirm that the uracil marker was incorporated in the MNN1 region of the chromosome in order to obtain the TIY1 strain.

Selective culture of the strain was carried out in a 5-FOA-containing YSD culture medium [1% yeast extract, 2% glucose, adenine (40 mg/L) and uracil (20 mg/L)] and a strain with the URA3 gene deleted was obtained. The PCR method was used similarly to the above procedures to confirm an mnn1-destroyed strain with the URA3 gene deleted. The strain containing mnn1::hisG was called a TIY3 strain.

The MNN1-destroyed strains are known to have mobility invertase, receiving N-bond modifications faster than the wild strain because the mannose of the α –1,3 bond at the nonreducing terminal is deleted. The wild strain and the TIY3 strain cultured in a YPAD culture medium were resuspended in a 0.2% sucrose-containing nutrient culture medium [1% yeast extract solution, 2% Bacto-Peptone, adenine (40 mg/L)], and cultured for 3 h. After recovery, the biomass was suspended in a SDS sample buffer solution, pulverized with glass beads, and 6% SDS-polyacrylamide electrophoresis was carried out using the supernatant. The invertase was detected after sucrose induction by carrying out active staining using triphenyltetrazolium [Ballou, Methods Enzymol., 185, 440-470 (1990)]. As a result, the mobility of the invertase produced by the TIY3 strain was confirmed to be faster than that of the wild strain.

(2) Preparation and properties of the mnn1 mnn4 auxotrophic double variant

The MNN4 gene is located in the No. 11 chromosome of yeast, and the DNA base sequence of the MNN4 gene is registered in the GenBank database as D83006 [Odani, et al., Glycobiology 6: 805-810 (1996)]. The 3' region of the MNN4 gene was amplified using PCR with primer E (AGATGCATACTAGTGGGCCCATTGTGATTGGAAT: sequence No. 5) and primer F (CCCCCGAATTCGTGTGAAGGAATAGTGACG: sequence No. 6), and the 5' region was amplified with primer G (CCCCCGAATTCAAGTCGGAGAACCTGACTG: sequence No. 7) and primer H (ATGGGCCCACTAGTATGCATCTCGCGTGGCATGG: sequence No. 8). These DNA fragments were incorporated into the HUH cassette-containing pSP73-HUH plasmid at the EcoRI site to prepare pSP73- mnn4::HUH. The plasmid was cleaved at the SpeI site to obtain a straight chain, and the TIY3 strain was transformed using the lithium acetate method. After transformation, it was sown on a SD-Ura culture medium plate, and cultured at 30°C for 2 days to obtain a transformant.

The genomic DNA of the transformant was prepared, the PCR method was carried out to confirm the incorporation of the uracil marker into the MNN4 region of the chromosome, and the strain was named TIY9.

The selective culture of the strain was carried out in 5-FOA-containing YSD culture medium, and a strain with the URA3 gene deleted was obtained. The PCR method was used

similarly to the above procedures to confirm a mnn4-destroyed strain with the URA3 gene deleted. The strain containing mnn1::hisG mnn4::hisG was called TIY11.

The presence of any phosphate group in the saccharide chains can be confirmed by carrying out staining with Alcian blue. Alcian blue is positively charged, and it binds to sites with a negative charge. Therefore, if yeast cells are suspended in a buffer solution at pH 3, and a 0.1% solution of Alcian blue 8GX (Sigma, code No. A3157) is added, only those cells having any phosphate groups in their saccharide chains are stained blue, and those which do not have any phosphate group remained unstained. The cell surface layer saccharide chain of the mnn4-destroyed strain has hardly any phosphate groups, and consequently there is no staining with Alcian blue. The wild strain, TIY3 strain and TIY11 strain were respectively cultured in nutrient culture medium, the cells were stained with Alcian blue, and as a result only the TIY11 strain was confirmed to be unstained.

(3) Preparation and properties of the mnn1 mnn4 och1 auxotrophic triple variant

The OCH1 gene is located in the No. 7 chromosome of yeast, and the DNA base sequence of the OCH1 gene is registered in the GenBank database as D11095 [Nakayama, et al., EMBO J. 11: 2511-2519 (1992)]. The AatII-HpaI site of the OCH1 gene of an already constructed plasmid pBL-OCH1 containing the whole length of the OCH1 gene [Nakayama, et al., EMBO J. 11: 2511-2519 (1992)] was cleared, and a plasmid pBL- och1::HUH having a HUH cassette obtained from pNKY51 inserted with blunt ends was prepared. The plasmid was cleared with SalI and BamHI to excise a region containing och1::HUH, and TIY11 was transformed using the lithium acetate method. The strain with och1 destroyed showed a low osmotic pressure sensitivity, thus after transformation, it was sown on a plate of a 0.3M KCl-containing SD-Ura culture medium and cultured at 30°C for 2 days to obtain a transformant.

The genomic DNA of the transformant was prepared, the PCR method was carried out to confirm the incorporation of the uracil marker in the OCH1 region of the chromosome, and the strain was named a TIY17.

The selective culture of the strain was carried out in a 5-FOA and 0.3M KCl-containing YSD culture medium, and a strain with the URA3 gene deleted was obtained. The PCR method was used similarly to the above procedures to confirm an och1-destroyed strain with the URA3 gene deleted. The strain containing mnn1::hisG mnn4::hisG och1::hisG was called TIY19.

The international deposition of the auxotrophic triple variant TIY19 strain was carried out at the Agency of Industrial Science & Technology, National Institute of Bioscience &

Human-Technology (1-1-3 Higashi, Tsukuba-shi, Ibaraki-ken) with deposit No. FERM BP-6802 on July 27, 1999.

The mobility of the invertase of this TIY19 strain containing och1-destroyed is known to be faster than that of the wild strain, TIY3 strain and TIY11 strain. Therefore, to confirm this effect of saccharide chain length in och1-destroyed strains, invertase detection was carried out respectively for the wild strain, TIY3 strain, TIY11 strain and TIY19 strain using the method described above, and as a result the mobility was found to become faster in the order of the wild strain, TIY3 strain, TIY11 strain and TIY19 strain.

Application Example 2

Preparation and properties of the mnn1 mnn4 och1 alg3 auxotrophic quadruple variant

The ALG3 gene is located on the No. 2 chromosome of yeast, and the DNA base sequence of the ALG3 gene is registered in the GenBank database as Z35844 [Feldmann, et al., EMBO J. 13: 5795-5809 (1994)]. The 5' region of the ALG3 gene was amplified using PCR with primer I (GCGGCCGCGAGACCTGAATCTTCGACACGCAAGAAAAA: sequence No. 9) and primer J (GAATTCGCTTTCGAACAAAATCAAAAGGGGCATAAC: sequence No. 10), and the 3' region was amplified with primer K (GAATTCCTATCCACCAAACTCACAAGCAAGCA: sequence No. 11) and primer L (GCGGCCGCCGAGAGGGTGAACGGTGCTAACTCAGGATT: sequence No. 12). These DNA fragments were incorporated in the HUH cassette-containing pSP73-HUH plasmid at the EcoRI site to prepare pSP73-alg3::HUH. The plasmid was cleared at the NotI site to obtain a straight chain, and the TIY19 strain was transformed using the lithium acetate method. After transformation, it was sown on a SD-Ura culture medium plate, and cultured at 30°C for 2 days to obtain a transformant.

The genomic DNA of the transformant was prepared, the PCR method was carried out to confirm the incorporation of the uracil marker in the ALG3 region of the chromosome, and the strain was named YS134.

The selective culture of the strain was carried out in 5-FOA-containing SD culture medium, and a strain with the URA3 gene deleted was obtained. The PCR method was used similarly to the above procedures to confirm an alg3-destroyed strain with the URA3 gene deleted. The strain containing mnn1::hisG mnn4::hisG och1::hisG alg3::hisG was called YS134-4A.

The international deposition of the auxotrophic quadruple variant YS134-4A strain was carried out at the Agency of Industrial Science & Technology, National Institute of Bioscience &

Human-Technology (1-1-3 Higashi, Tsukuba-shi, Ibaraki-ken) with deposit No. FERM BP-6801 on July 27, 1999.

The saccharide chain length of this YS134-4A strain containing alg3-destroyed is short, and consequently the mobility of invertase is known to be faster than that of the wild strain, TIY3 strain, TIY11 strain and TIY19 strain. Therefore, to confirm the effects of saccharide chain length in this alg3-destroyed strain, invertase detection was carried out respectively for the wild strain, TIY3 strain, TIY11 strain, TIY19 strain and YS134-4A strain using the method described in Application Example 1 (1), and as a result the mobility was found to become faster in the order of the wild strain, TIY3 strain, TIY11 strain, TIY19 strain and YS134-4A strain.

Application Example 3

Isolation of the cell surface layer mannan protein from the mnn1 mnn4 och1 auxotrophic triple variant and structural analysis of saccharide chains contained in it.

Concanavalin A is a lectin showing affinity to a saccharide chain containing 2 or more of -D-Man residues without substitution of the hydroxyl groups at the C-3, C-4 and C-6 positions and, by immobilizing it on a column, it is possible to separate mannan protein from glucan, which is a yeast cell wall polysaccharide of chitin, etc. First of all, the mannan protein of the cell surface layer was isolated from the TIY19 strain biomass [Peat, et al., J. Chem. Soc. 29 (1961)].

A 500-mL Sakaguchi flask was charged with 50 mL of 0.3M KCl-containing YPAD culture medium, cultured at 30°C for 24 h, the biomass was collected by centrifugation, suspended in 10 mL of a 100 mM sodium citrate buffer solution (pH 7.0) and heated at 121°C in an autoclave for 1 h. After cooling, the mixture was centrifuged, the supernatant was obtained, the centrifugation cake was suspended in 10 mL of water, heated, centrifuged, and the supernatant was collected. All of the extracts were combined, and the combined solutions were poured into 3 times their volume of ethanol. The white precipitate formed was collected and dried. The product was dissolved in a concanavalin A (ConA) column buffer solution [0.1M sodium phosphate buffer solution (pH 7.2) containing 0.15M sodium chloride and 0.5 mM calcium chloride], the solution was applied to a ConA-agarose column (0.6 x 2 cm, Honen Corporation), and after washing with the ConA column buffer solution, it was eluted with the ConA column buffer solution containing 0.2M -methylmannoside. The fraction collected was dialyzed and freeze-dried to obtain mannan proteins.

Enzymatic treatment of the mannan protein prepared was carried out to cleave its Asn-bound saccharide chain. Specifically, the freeze-dried standard product was dissolved in 100 μL of a buffer solution for N-glycosidase F [0.5% SDS, 0.35% 2-mercaptoethanol-containing 0.1M Tris-HCl buffer solution (pH 8.0)], and the solution was

boiled for 5 min. After cooling to room temperature, 50 μL of 7.5% Nonidet P-40, 138 μL of H_2O, 12 μL of N-glycosidase F (Bellinger Co. [transliteration]) were added, and the mixture was processed at 37°C for 16 h. After desalting with a BioRad AG501-X8 column, an equal volume of a mixture of phenol:chloroform (1:1) was added, the mixture was vigorously shaken, and the surfactant and protein were removed to obtain a saccharide chain preparation.

The following procedures were carried out fluorescence-labeling (pyridyl amination, PA) of the saccharide chains prepared. After concentration of the saccharide chain preparation to dryness, 40 μL of a coupling reagent (552 mg of 2-aminopyridine dissolved in 200 μL of acetic acid) were added, the container containing the mixture was sealed and treated at 90°C for 60 min. After cooling to room temperature, 140 μL of a reducing agent (200 mg of a borane-dimethylamine complex dissolved in 50 μL of H_2O and 80 μL of acetic acid) were added, the container containing the mixture was sealed and treated at 80°C for 80 min. After the reaction, 200 mL of aqueous ammonia were added, an equal volume of a mixture of phenol:chloroform (1:1) was added, the mixture was shaken vigorously, and the aqueous phase containing the PA-oligosaccharides was recovered. This procedure was repeated 7 times, and the 2-aminopyridine not consumed was removed. The supernatant was filtered through a 0.22 μm filter to obtain a PA-oligosaccharide preparation.

Using an amide column for HPLC, PA-oligosaccharides can be separated depending on their chain length. The column used was a TSKGel Amide-80 (4.6 x 250 mm, Tosoh), and as a solvent, a 35:65 mixture (solvent A) of a 200 mM acetic acid-triethylamine buffer solution (pH 7.0) and acetonitrile and a 50:50 mixture (solvent B) of a 200 mM acetic acid-triethylamine buffer solution (pH 7.0) and acetonitrile were prepared.

The column was equilibrated in advance by allowing solvent A to flow at a rate of 1.0 mL/min, the proportion of the solvent B was increased linearly immediately after sample injection to 50% over 25 min, and subsequently solvents A and B were allowed to flow at 50:50 for 5 min, allowing the PA-oligosaccharides to elute. Figure 6 shows the results obtained. The saccharide chains of mannan proteins produced by the TIY19 strain, which is a och1 mnn1 mnn4 auxotrophic triple variant, showed mostly a single peak from the amide column. The elution position of the peak coincided with that of a $Man_8GlcNAc_2$-PA standard product (Takara Shuzo). Therefore, the mannan protein produced by the TIY19 strain was found to have a high mannose saccharide chain $Man_8GlcNAc_2$ added.

Application Example 4

Insertion of the -mannosidase I gene into the och1 mnn1 mnn4 auxotrophic triple variant

To allow yeast to carry out the biosynthesis of a saccharide chain identical to a corresponding mammalian type, the -mannosidase I gene (-1,2-mannosidase) may be inserted into the auxotrophic triple variant for expression. As a result, the biosynthesis of $Man_8GlcNAc_2$, which is a mammalian composite or complex precursor can be carried out.

The TIY19 strain was transformed using the lithium acetate method with an endoplasmic reticulum expression plasmid pGAMH1 for the -1,2-mannosidase of *Aspergillus saitoi*-origin with the actual expression results previously discovered [Chiba, et al., J. Biol. Chem. 273: 26298-26304 (1998)]. As a control, the strain was transformed using only vector pG3 containing no -1,2-mannosidase gene. After transformation, it was sown on a plate containing SD-Trp culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding tryptophan (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The transformant prepared was called TIY19pGAMH1.

The transformant prepared was used to prepare a saccharide chain using the same procedures as those used in Application Example 3, and the HPLC analyses were carried out.

The results of the analyses with an amide column are shown in Figure 7. In the case of a control with the vector alone, there was mostly a single peak (Figure 7, a) similarly to the result of Application Example 3, and the elution position was found to coincide with that of a $Man_8GlcNAc_2$-PA standard product (Takara Shuzo). On the other hand, in the case of TIY19pGAMH1 containing the -1,2-mannosidase gene, there were mostly 4 peaks observed (Figure 7, b). These peaks showed the same elution positions as those of the $Man_5GlcNAc_2$-PA, $Man_6GlcNAc_2$-PA, $Man_8GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA standard products, in the order of elution. They are called human high mannose saccharide chains.

The $Man_5GlcNAc_2$-PA fraction showing the fastest elution was collected and subsequently applied to a reverse phase column.

In the case of HPLC using a reverse phase column, it is possible to separate PA-oligosaccharides according to their structures. The column used was TSKGel ODS-80T_M (4.6 x 150 mm, Tosoh), and as a solvent, a 100 mM ammonium acetate buffer solution (pH 4.0) (solvent A) and a 100 mM ammonium acetate buffer solution (pH 4.0) containing 0.5% 1-butanol (solvent B) were prepared.

A 95:5 mixture of solvents A and B was allowed to flow at a flow rate of 1.2 mL/min to equilibrate the column in advance. The proportion of the solvent B was increased linearly immediately after sample injection to 50% over 20 min, allowing the PA-oligosaccharides to elute. The results obtained are shown in Figure 8. There was only one peak in the saccharide chain fraction obtained from the reverse phase column (Figure 8, a), and the elution position of

the peak coincided with that of the $Man_5GlcNAc_2$-PA standard product (Takara Shuzo) having a structure represented by the following formula (III);

//insert the formula (III)//

(where Man denotes mannose, GlcNAc denotes N-acetylglucosamine, and # shows a GnT-I active site) (Figure 8, b). Therefore, the mannan protein produced by the TIY19pGAMH1 strain was found to contain a $Man_5GlcNAc_2$ saccharide chain, which is a hybrid or complex chain precursor.

Application Example 5

Synthesis of a hybrid saccharide chain ($GlcNAcMan_5GlcNAc_2$) standard product

To confirm the biosynthesis of a hybrid saccharide chain ($GlcNAcMan_5GlcNAc_2$), the synthesis of the desired saccharide chain was carried out by carrying out the enzymatic reaction of GnT-I outside of the cell. GnT-I shows very strict substrate specificity, and in the case of a saccharide chain structure represented by formula (III), it allows transfer of GlcNAc with -1,2-bonding to only a mannose residue at the position marked with # in the formula.

The expression of the rat GnT-I gene in yeast was successfully achieved by Yoshida, et al. [Yoshida, et al., Glycobiology 9: 53-58 (1999)]. This gene was ligated downstream of a GAP-DH promoter in pG3, which is a multicopy plasmid, and subsequently scission with SmaI-NaeI was carried out, excising the promoter and the subsequent region containing an ORF terminator of GnT-I. Then, the fragment was inserted at the SmaI site of PY0354, which is a multicopy plasmid. The plasmid prepared was called pYOG4. A wild yeast strain YPH500 was transformed using the plasmid and lithium acetate method. After transformation, it was sown on a plate containing SD-Trp culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding tryptophan (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The transformant prepared was called YPH500/pYOG4.

Subsequently, liquid culture was carried out in 500 mL of a SD-Trp culture medium solution [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding tryptophan (20-400 mg/L)], and the biomass was collected. After washing with cold water, the biomass was suspended in 5.7 mL of a spheroplast culture medium [50 mM potassium phosphate (pH 7.5) containing 1M sorbitol], 9 μL of 2-mercaptoethanol and

12 mg of Zymolyase 100T dissolved in 300 μL of the spheroplast culture medium was added, and the mixture was held at 30°C for 45 min. After adding 15 mL of 1M sorbitol and carrying out centrifugation, the pellet was washed with 15 mL of 1M sorbitol and centrifuged. To the pellet, 4 mL of a lysis buffer solution [250 mM sorbitol, 2 μg/mL of antipine, 2 μg/mL of chymostatin, 3 μg/mL of leupeptin, 3 μg/mL of pepstatin, 1 mM benzamidine, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF-containing 10 mM triethanolamine solution (pH 7.2)] was added, the cell was pulverized in a homogenizer, the homogenized mixture was centrifuged at 220 G, and the supernatant was recovered. The supernatant was centrifuged further at 100,000 G, the pellet was suspended in 150 μL of the lysis buffer solution to obtain a GnT-I enzyme solution. Incidentally, no other GnT activity was detected in this standard product.

Subsequently, the synthesis of the desired saccharide chain was carried out. A PA-labeled $Man_5GlcNAc_2$ saccharide chain (purchased from Takara Shuzo) was used as a receptor substrate and placed in a 200 pmole tube. After evaporation to dryness, 8.2 μL of the GnT-I enzyme solution prepared as described above, 2 μL of 0.2M $MnCl_2$ and 9.8 μL of a GnT-I reaction buffer solution [0.17M MES (pH 6.0), 1.7% Triton X-100, 0.34% bovine serum albumin, 8.47 mM AMP, 1.69 mM UDP-GlcNAc and 169 mM GlcNAc] were added to the tube, and the reaction was carried out at 37°C for 3 h. After stopping the reaction by boiling for 5 min, the reaction mixture was filtered and applied to HPLC.

The column used was TSKGel ODS-80T_M (4.6 x 250 mm, Tosoh), and as a solvent, a 100 mM ammonium acetate buffer solution (pH 6.0) containing 0.15% 1-butanol was used. The column was equilibrated in advance by allowing the solvent to flow at a flow rate of 1.2 mL/min, a sample was injected, and the PA-oligosaccharides were eluted. Figure 9 shows the results obtained. The reaction mixture showed mostly 2 peaks on the reverse phase column used, and the elution position of the peak which eluted first was found to coincide with that of a $Man_5GlcNAc_2$-PA standard product [Takara Shuzo, showing a structure represented by the formula (III) shown above]. Therefore, it was considered to be the receptor substrate not consumed in the reaction.

On the other hand, the peak coming out later was collected, and after purification the mass analysis was carried out using TOF-MS. A ThermoQuest LASERMAT2000 was used, and the analyses were carried out with a 0.01% solution of disodium phosphate containing 2.5% of 2,5-dihydroxybenzoic acid and 40% acetonitrile. As a result, the mass of the peak fraction was found to be the molecular weight predicted [m/z = 1521 (H^+); m/z = 1541 (Na^+)]. Because of the strict substrate specificity of GnT-I, the saccharide chain obtained was considered to be the desired hybrid saccharide chain $GlcNAcMan_5GlcNAc_2$ having a structure represented by the following formula (IV).

//Insert the formula (IV).//

(In the formula, Man denote mannose, and GlcNAc denotes N-acetylglucosamine.)

Application Example 6

Insertion of the -mannosidase I and GnT-I genes into the och1 mnn1 mnn4 auxotrophic triple variant

To allow yeast to carry out biosyntheses of hybrid saccharide chains, the GnT-I gene may be inserted into the yeast strain prepared in Application Example 4 for expression. As a result, the biosynthesis of a mammalian hybrid $GlcNAcMan_5GlcNAc_2$ saccharide chain becomes possible.

The endoplasmic reticulum expression plasmid pGAMH1 for *Aspergillus saitoi*-origin -1,2-mannosidase [Chiba, et al., J. Biol. Chem. 273: 26298-26304 (1998)] used in Application Example 4 was cleared with SmaI-NaeI to excise the promoter and the region containing the ORF terminator of -1,2-mannosidase which followed. The fragment was inserted at the SmaI site of pYOG4. The plasmid prepared was named pYOMG4. The TIY19 strain was transformed with the plasmid using the lithium acetate method. As a control, pYO354 alone was used for transformation. After transformation, it was sown on a plate of a SD-Trp culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding tryptophan (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The transformant prepared was called TIY19pYOMG4.

The international deposition of the auxotrophic triple variant TIY19pYOMG4 strain having both the -1,2-mannosidase [sic] and GnT-I genes inserted and producing a hybrid saccharide chain was carried out at the Agency of Industrial Science & Technology, National Institute of Bioscience & Human-Technology (1-1-3 Higashi,, Tsukuba-shi, Ibaraki-ken) with deposit No. FERM BP-6775 on July 2, 1999.

The transformant prepared was used to prepare a saccharide chain similarly to Application Example 3, and the saccharide chain prepared was analyzed by HPLC.

Figure 10 shows the results of analyses carried out with an amide column. In the control sample prepared with the vector alone, there was mainly one peak, similar to the result obtained in Application Example 3, and the elution position was found to coincide with that of a $Man_8GlcNAc_2$-PA standard product (Takara Shuzo) (Figure 10, A). In the case of

TIY19pYOMG4, containing both the -1,2-mannosidase and GnT-I genes, 5 major peaks were observed (Figure 10, B). The elution positions of four of the peaks (Figure 10, B: peaks a, c, d and e) were found to coincide those of the $Man_5GlcNAc_2$-PA, $Man_6GlcNAc_2$-PA, $Man_7GlcNAc_2$-PA and $Man_8GlcNAc_2$-PA standard products. They are called human high mannose saccharide chains. Furthermore, a new peak (Figure 10, B: peak b), not observed when the -1,2-mannosidase gene alone was inserted, was developed. The elution position of this peak was found to coincide with that of the hybrid $GlcNAcMan_5GlcNAc_2$ saccharide chain standard product prepared in Application Example 5. The peak fractions were collected and applied to a reverse phase column similarly to Application Example 3. The same conditions for the column and solvent were used as those shown in Application Example 5. The fraction showed only one main peak from the reverse phase column used (Figure 11), and the elution position of the peak was found to coincide with that of the $GlcNAcMan_5GlcNAc_2$-PA standard product. Therefore, the mannan protein produced by the TIY19pYOMG4 strain was also found to contain a hybrid $GlcNAcMan_5GlcNAc_2$ saccharide chain.

Application Example 7

Expression of human hepatic -mannosidase II in yeast

This -mannosidase II is an enzyme for conversion of a hybrid saccharide chain to a single chain complex saccharide chain inside the Golgi body.

The sequence of the human hepatic -mannosidase II gene has been registered in the GenBank database as U31520 [Misago, et al., Proc. Natl. Acad. Sci., USA 92: 11766-11770 (1995)]. The Clonetech human liver Marathon-Ready cDNA was used as a template, and PCR amplification was carried out for the portion coding for the N-terminal region of -mannosidase II using primer M (CGCCGCCGAGCTCTAAAAAAATGAAGTTAAGCCGCC: sequence No. 13) and primer N (ATCCCACCACTTTGAAAGGT: sequence No. 14); the portion coding for the center using primer O (GAAGACTCACGGAGGAAGTT: sequence No. 15) and primer P (ATGGCGGTATATGTGCTCGA: sequence No. 16); and the portion coding for the C-terminal region using primer Q (CGCAGTTTGGGATACAGCAA: sequence No. 17) and primer R (ATTATTATTAGCGGCCGCCCCTCAACTGGATTCG: sequence No. 18). The DNA fragments obtained were inserted at the Srfl site of pCRScript, and after confirming the sequence, insertion was changed to a Bg1II site in order to obtain a correct sequence coding for every region. The plasmid prepared was named pCRMAN2.

To confirm the expression of the desired protein, a gene with 3 repeats of the 30 bp HA tag coding for the influenza virus hemaglutinin epitope nonligated to the 3' terminal of the -mannosidase II gene in order to construct a vector. Specifically, double-strand DNA

comprising sequence S (sequence No. 19) was chemically synthesized and inserted between the BamHI and EcoRI sites of expression plasmid pYEX-BX. The plasmid obtained was named pYEX-BX-3HA. Subsequently, the portion coding for -mannosidase II was excised from pCRMAN2 using BamHI and EcoRI and inserted between the SacI and NotI sites of pYEX-BX-3HA. This plasmid was named pYEMAN2-HA.

Subsequently, to improve the amount of expression in yeast, the portion coding for the membrane piercing region of -mannosidase II was substituted with that of the gene (OCH1) coding for the -1,6-mannosyl transferase of yeast. Specifically, double-stranded DNA comprising sequence T (sequence No. 20) was chemically synthesized and inserted between the SacI and EcoRI sites of pBluescript. This plasmid was called pBOCH1. On the other hand, pYMAN2-HA was used as a template, and the amplification of part of the portion coding for the catalytic region of -mannosidase II was carried out using primer U (TTAGACTACCCATGGAACCCGCGCCGCGAGGGCTCCTTC: sequence No. 21) and primer V (CAGGAGAACTTTGGTTCGAAAAAGCTTTGACTTCTT: sequence No. 22). After confirming the sequence, scission with NcoI and HindIII and insertion between NcoI and HindIII of pBOCH1 was carried out. Subsequently, fragments were excised from this plasmid with SacI and PstI and the portion between SacI and PstI of pYEMAN2-HA was substituted. The plasmid was named pYEOM2-HA.

As a host, the *S. cerevisiae* yeast wild strain YPH500 was used, and the lithium acetate method was used to carry out the transformation. As a control, pYEX-BX-3HA was used. After transformation, it was sown on a plate of SD-Ura culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding uracil (20-400 mg/L)], and cultured at 30°C for 2 day to obtain a transformant.

The yeast transformed was cultured at 30°C in the SD-Ura culture medium until the OD660 = 0.8, a suitable amount of copper sulfate was added, and the culture was continued for 2 h. After collecting the biomass, the cells were pulverized in an SDS sample buffer solution using glass beads, and Western blot analyses were carried out using the cell extract. The Western blot analyses were carried out using rat anti-HA antibody as a primary antibody and an anti-rat IgG antibody-peroxidase complex as a secondary antibody, and detection was carried out by slight exposure on an X-ray film using Super Signal Ultra as a substrate. As a result, no signal at all was observed in the control, whereas in the extract of the cells transformed with pYEOM2-HA, a signal was confirmed at a position of a molecular weight of about 140,000 (Figure 12).

Subsequently, the hybrid saccharide chain prepared in Application Example 5 [having a structure like that shown in formula (IV)] was used as a substrate, and the enzymatic activity of

-mannosidase II was measured. In a sample tube, 100 pmole of the hybrid saccharide chain [having a structure like that shown in formula (IV)] was dried, Subsequently, 2 μL each of 0.2M $MnCl_2$, 1M GlcNAc and 1M sodium acetate buffer solution (pH 5.6) and 8 μL of H_2O were added to the tube, and 8 μL of the cell extract was added to initiate the enzymatic reaction. After holding overnight at 37°C, the reaction mixture was boiled to stop the reaction, any insoluble fraction was removed by centrifugation, and the analyses with HPLC were carried out. The same HPLC conditions as those of Application Example 5 were used. As a result, if the cell extract of yeast with -mannosidase II expression was used as an enzyme source, the peak at 40 min clearly increased from that of the control (Figure 13, B). This peak at 40 min was found to coincide with the elution position of a single strand complex saccharide chain represented by the following formula (V) obtained from the product of enzymatic digestion of a PA saccharide chain standard product (Takara Shuzo PA-Sugar Chain 022) [Oguri, et al., J. Biol. Chem. 272: 22721-22727 (1997)], and thus, it was confirmed to be the activity of -mannosidase II.

//Insert the formula (V).//

(In the formula, Man denotes mannose, and GlcNAc denotes N-acetylglucosamine.)

Application Example 8

Preparation of an auxotrophic triple variant with the genes necessary for the production of a double strand complex saccharide chain inserted

The expression of human GnT-II in yeast has been reported by Yoshida, et al. [Yoshida, S., et al., Abstracts of the meeting on yeast cell biology, p. 279, Cold Spring Harbor Laboratory (1997)]. The GnT-II gene region, including the promoter, was excised from an expression vector pSY114-GnT-II using XbaI and inserted at the XbaI site of pBluescript SK. The plasmid was named pBlueGT2. Subsequently, the GnT-I gene region including the promoter was excised from the plasmid pYOG4 shown in Application Example 6 using BamHI and XbaI and inserted at the BamHI and XbaI sites of pBlueGT2. The desired fragment was excised from this plasmid using BssHII, and after blunting the ends with the T4 DNA polymerase, the fragment was inserted at the SmaI site of a pASZ10 plasmid [Stotz and Linder, Gene 95: 91-98 (1990)] having ADE2 as a marker. This plasmid was named pASZGN12. The plasmid pASZGN12 was made into a straight chain using HpaI and used to transform the auxotrophic triple variant TIY19 strain

prepared in Application Example 1 using the lithium acetate method. After transformation, it was sown on a plate containing 0.3M KCl in SD-Ade culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding adenine (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The genomic DNA of the transformant was obtained, the PCR method was used to confirm that GnT-I and GnT-II genes were incorporated into the chromosome in the ADE2 region to obtain the YCY22 strain. The cell extract of the YCY22 strain was obtained, the enzymatic activity was respectively measured, and the expression of GnT-I and GnT-II was confirmed.

On the other hand, the expression of human β-1,4-GalT in yeast has been reported by Yoshida, et al. [Yoshida, S., et al., Abstracts of the meeting on yeast cell biology, p. 279, Cold Spring Harbor Laboratory (1997)]. The β-1,4-GalT gene region, including the promoter, was excised from the expression vector pGalT13C using SalI and XhoI and inserted at the SalI, XhoI site of pRS403. The plasmid was named pRSGAL1. Furthermore, the expression of the human UDP-Gal transporter (Ugt2p) in yeast has been reported by Kainuma, et al. [Kainuma, et al., Glycobiology 9:133-141 (1999)]. From the expression plasmid YEp352-GAP-UGT2 of this gene (UGT2), the gene region including the promoter was excised using BamHI and inserted at the BamHI site of pRSGAL1. This plasmid was named pRSGATP1. The plasmid pRSGATP1 was made into a straight chain with NdeI, and the YCY22 strain was transformed using the lithium acetate method. After transformation, it was sown on a plate containing 0.3 M KCl SD-His culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding histidine (20-400 mg/L)], and the cultivation was carried out at 30°C for 2 day to obtain a transformant. The genomic DNA of the transformant was prepared, and the PCR method was used to confirm that the β-1,4-GalT and Ugt2 genes were inserted in the HIS3 region of the chromosome, and the strain was named the YCY42 strain. The cell extract of the YCY42 strain was used to measure the respective enzymatic activities to confirm the expression of β-1,4-GalT and Ugt2p.

The gene fragment including HA-tag was excised from the human liver α-mannosidase II expression vector pYEOM2-HA using SacI and SphI, and the ends were blunted with T4 DNA polymerase. The fragment was inserted at the SmaI site of pAUR123. After the promoter was ligated in the correct direction, the α-mannosidase II gene region, including the promoter, was excised using BamHI and inserted at the BamHI site of pRS406. This plasmid was made into a straight chain with NdeI, and the above YCY42 strain was transformed using the lithium acetate method. After transformation, it was sown on a plate containing 0.3M KCl in SD-Ura culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding uracil (20-400 mg/L)], and the culture medi was carried out at

30°C for 2 days to obtain a transformant. The genomic DNA of the transformant was prepared, and the PCR method was used to confirm that the URA3 region of the genes was inserted into the chromosome, and the strain was named the YCY52 strain. The cell extract of the YCY52 strain was used to measure the enzymatic activity in order to confirm the expression of α-mannosidase II

The α-1,2-mannosidase gene fragment including the promoter region was excised from the α -1,2-mannosidase expression vector pGAMH1 shown in Application Example 4 with NaeI and SmaI and inserted in the SmaI site of the pYO325 vector. This plasmid was named pYOM5. Furthermore, the UDP-GlcNAc transporter gene necessary for supplying the substrate to the Golgi body was inserted. The expression of the human UDP-GlcNAc transporter gene in yeast has been reported by Ishida, et al., [Ishida, et al., J. Biochem. 1261: 68-77 (1999)]. This expression vector was used as a template, and the PCR method was carried out to amplify the UDP-GlcNAc transporter with primer W (AGAGCGCCGCAAAATGTTCGCCAACCTAA: sequence No. 23) and primer X (TTTTGTCGACTAGACGCGTGAAGCATGCCC: sequence No. 24). After confirming the sequence, scission was carried out with NotI and SalI, and the portion between the NotI and SalI sites of pG3-N was substituted. Subsequently, the UDP-GlcNAc transporter gene fragment containing the promoter region was excised from the plasmid with NaeI and SmaI and inserted at the SmaI site of pYOM5. The plasmid was named pYOMR. The plasmid prepared was used to transform the YCY52 strain described above using the lithium acetate method. After transformation, it was sown on a plate containing 0.3M KCl in SD-Leu culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding leucine (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The transformant prepared was named the YCY73 strain. The cell extract of the YCY73 strain was used to measure the enzymatic activities, and as a result the expression of α -1,2-mannosidase and UDP-GlcNAc transporter was confirmed.

Furthermore, a plasmid was prepared for integration of msdS and hUGTre12. The msdS sequence, including the GAP promoter of PGAMH, was excised with SmaI and NaeI and inserted at the PvuII site of pRS404. The plasmid prepared was named msdS-pRS404. The hUGTre12 sequence, including the GAP promoter, was excised from the plasmid hUGTre12-pG3 having hUGTre12 inserted downstream of the GAP promoter with SmaI and NaeI and inserted at the PstI site of msdS-pRS404. The plasmid prepared was named HM-pRS404. BstXI was used to clean HM-pRS404 within TRP1, and the YCY42 strain was transformed using the lithium acetate method. The transformant was cultured with 5 mL of YPAD + 0.3M KCl at 30°C for 2 days, and the PCR method was carried out to confirm that

madS and hUGTre12 were incorporated into the TRP1 chromosome. Furthermore, the cell extract was used to measure the enzymatic activities, and the expression of α -1,2-mannosidase and UDP-GlcNAc transporter was confirmed. The strain with msdS and hUGTre12 integrated into the YCY42 strain was named the TIY63 strain.

The human hepatic α -mannosidase II-expression vector pYEOM2-HA was used to excise the gene fragment, including HA-tag, using SacI and SphI, and the ends were blunted with T4 DNA polymerase. The fragment was inserted at the SmaI site of pAUR123. After confirming that the promoter was ligated in the correct direction, the α -mannosidase II gene region, including the promoter, was excised with BamHI and inserted at the BamHI site of pRS406. The plasmid was made into a straight chain with NdeI, and the TIY63 strain was transformed using the lithium acetate method. After transformation it was sown on a plate containing 0.3M KCl in SD-Ura culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding urasil (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The genomic DNA of the transformant was prepared, the PCR method was carried out to confirm that the gene was incorporated into the URA3 region of the chromosome, and the strain was named MSY3. The enzymatic activity was measured using the cell extract of the MSY3 strain to confirm the expression of α -mannosidase II.

Application Example 9

Preparation of auxotrophic quadruple variant with the gene necessary for the production of a double strand complex saccharide chain inserted

First of all, the plasmid pASZGN12 prepared in Application Example 8 was made into a straight chain with HpaI, and the transformation of the auxotrophic quadruple variant YS134-4A strain prepared in Application Example 2 was carried out using the lithium acetate method. After transformation, it was sown on a plate containing 0.3M KCl in SD-Ade culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding adenine (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The genomic DNA of the transformant was prepared, the PCR method was carried out to confirm that the GnT-I and GnT-II genes were incorporated into the ADE2 region of the chromosome, and the strain prepared was named YCY122. The cell extract of the YCY122 strain was used to measure the enzymatic activities to confirm the expression of GnT-I and GnT-II.

Subsequently, the plasmid pRSGATP1 prepared in Application Example 8 was made into a straight chain using NdeI, and the YCY122 strain was transformed using the lithium acetate method. After transformation, it was sown on a plate containing 0.3M KCl in SD-His culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and

amino acid mixture excluding histidine (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The genomic DNA was prepared from the transformant, the PCR method was carried out to confirm that the α -1,4-GalT and UGT2 genes were incorporated into the HIS3 region of the chromosome, and the strain obtained was named YCY142. The cell extract of the YCY142 strain was used to measure the enzymatic activities, and the expression of β-1,4-GalT and Ugt2p was confirmed.

Furthermore, the plasmid pYOMR5 prepared in Application Example 8 was used to transform the YCY142 strain using the lithium acetate method. After transformation, it was sown on a plate containing 0.3M KCl in SD-Leu culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding leucine (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The strain obtained was named YCY163. The cell extract of the YCY163 strain was used to measure enzymatic activities, and the expression of α-1,2-mannosidase and UDP-GlcNAc transporter was confirmed.

The lectin-stainability of the YCY163 strain prepared was evaluated in order to examine any changes in the saccharide chain structure of the mannan protein on the cell surface layer of the yeast. Concanavalin A is known to bind to a high mannose, hybrid or double strand complex saccharide chain containing 3 specific mannose residues, and its affinity is known to be higher in a high mannose saccharide chain compared to a hybrid or double strand mixed saccharide chain. Therefore, the yeast cells collected were mixed with Texas red-labeled concanavalin A solution, and the mixture was maintained at 4°C for 2 h while stirring occasionally. After washing with PBS and subsequently with PBS containing 10 mM α-methylmannoside, and the cells were observed under a fluorescent microscope. As a result, the YS134-4A strain was observed to be stained fluorescence even after washing, and the YCY163 strain was confirmed to show fluorescence at the periphery of the cell at an apparently reduced intensity. Therefore, in the YCY163 strain, the amount of a high mannose saccharide chain was reduced, and the complex saccharide chain was formed.

Application Example 10

Production of human fibroblast growth factor (FGF) and saccharide chain structure modification in a yeast strain having the ability to produce mammalian saccharide chains

The FGF6-1 chimeric gene [secFGF(N35)] was a gift from Ms. Atuko Yoneda of the National Institute of Bioscience and Human Technology [Yoneda, et al., BioTechniques, 27:576-590 (1999)]. FGF was excised from SecFGF(N35)/pBS using SmaI and NaeI and inserted at the HindIII position of pGEM2-α36. The plasmid prepared was named pFGFα23. The

prepro α-factor and FGF region were cut out by carrying out the scission of pFGFα23 with EcoRI and inserted at the EcoRI site of the pUC119 plasmid. The plasmid prepared was named FGF-pUC119. To remove the EAEA sequence of the α-factor, PCR amplification was carried out using primer Y (CGCCAGGGTTTTCCCAGTCACGAC: sequence No. 25) and primer Z (ATGGGCCGGCTCTTTTATCCAAAGATAC: sequence No. 26). The DNA fragment was incorporated at the EcoRI site of pUC18 to obtain plasmid pAF02. FGF was excised from pFGF01 using NaeI and SmaI and inserted at the NaeI and SmaI sites of pAF02. The plasmid prepared was named pAF03. The scission of pAF03 with EcoRI was carried out to excise the prepro α-factor and FGF region, which were incorporated downstream of the GAP promoter of YEp352GAP plasmid to obtain plasmid pAFF2. Subsequently, pAFF2 was cleared with AatII and HpaI to excised the 2 μm region in order to construct plasmid pAFF3 for yeast integration. Subsequently, the scission of pAFF3 with ApaLI and AccI was carried out to excise the GAP promoter and the FGF sequence and inserted at the PvuII site of plasmid pYO325 having a LEU2 marker. Furthermore, the 2 μm region of the plasmid was removed by excision SpeI. The plasmid prepared was named pAFF9. The yeast strains (TIY19 and YCY42) were transformed after making pAFF9 a straight chain by cleavage with EcoRV using the lithium acetate method. After transformation, it was sown in SD-Leu culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding leucine (20-400 mg/L)], and cultured at 30°C for 2 days to obtain the respective transformants.

The transformants were cultured in 5 mL of YPAD + 0.5M KCl at 30°C for 3 days, 50 μL bed of heparin-Sepharose suspension (Pharmacia) was added to the culture supernatant, and the mixture was shaken overnight at 4°C to allow FGF to be adsorbed on the heparin-Sepharose. Subsequently, the heparin-Sepharose was recovered by centrifugation, and after boiling in the SDS sample buffer solution, the supernatant was applied to SDS-PAGE. Western blot was carried out using an anti-FGF antibody, confirming the expression of FGF. Furthermore, the PCR method was used to confirm that FGF was incorporated at the LEU2 site of the chromosome, the TIY19 strain with FGF integrated was named TIY48, and the YCY42 strain with FGF integrated was named TIY49.

To achieve stable and efficient expression of the proteins, a plasmid for integration of msdS was prepared. The scission of plasmid pGAMH having msdS inserted downstream of the GAP promoter was carried out with EcoRI to prepare a plasmid with the 2 μm region removed. The plasmid prepared was named pImsdS. The scission of pImsdS inside TRP1 was carried out with XbaI, and TIY48 (Δmnn1::hisG, Δmnn4::hisG, Δoch1::hisG, FGF::LEU2) and TIY49 ((Δmnn1::hisG, Δmnn4::hisG, Δoch1::hisG, FGF::LEU2 ade::[GnT-I & GnT-II] his3::[β-1,4-GalT & UGT2]) were transformed using the lithium acetate method. The

transformants were cultured in 5 mL of YPAD + 0.5M KCl at 30°C for 3 days, 50 μL of heparin-Sepharose (Pharmacia) were added to the culture solution, and the mixture was shaken overnight at 4°C to allow FGF to adsorb onto the heparin-Sepharose. Subsequently, the heparin-Sepharose was recovered, and Western blot was carried out using a FGF antibody to confirm the expression of msdS. Furthermore, the PCR method was used to confirm that msdS was incorporated at the TRP1 site of the chromosome, the TIY48 strain with msdS integrated was named TIY53, and the strain of TIY49 strain with msdS integrated was named TIY54.

Subsequently, a plasmid for integration of msdS and hUGTre12 was prepared. The scission of PGAMH was carried out with SmaI and NaeI to excise the GAP promoter and msdS sequence, which were inserted at the PvuII site of pRS404. The plasmid prepared was named msdS-pRS404. The scission of plasmid hUGTre12-pG3 having hUGTre12 inserted downstream of the GAP promoter was carried out with SmaI and NaeI to excise the GAP promoter and hUGTre12 sequence, which were inserted at the PatI site of msdSpRS404. The plasmid prepared was named HM-pRS404. The TIY48 and TIY49 strains were transformed by cleaving HM-pRS404 inside TRP1 with BstXI and using the lithium acetate method. The transformants were cultured in 5 mL of YPAD + 0.3M KCl at 30°C for 3 days, 50 μL of a heparin-Sepharose suspension (Pharmacia) were added to the culture supernatant, and the mixture was shaken overnight at 4°C to allow FGF to adsorb to the heparin-Sepharose. Subsequently, the heparin-Sepharose was recovered centrifugation, and after boiling in the SDS sample buffer solution, the supernatant was applied to SDS-PAGE. Western blot was carried out using a anti-FGF to antibody confirm the expression of FGF. Furthermore, the PCR method was used to confirm that msdS and hUGTre12 were incorporated at the TRP1 site of the chromosome. The enzymatic activities were measured using the cell extracts to confirm the expression of α-1,2-mannosidase and UDP-GlcNAc transporter in both strains. The TIY48 strain with msdS and hUGTre12 integrated was named TIY59, and the TIY49 strain with msdS and hUGTre12 integrated was named TIY60.

To prepare saccharide chains, the TIY48 and TIY53 strains with FGF integrated at the LEU2 site of the chromosome were used to purify FGF from 3 L of the culture. To a culture solution prepared by culturing in 3 L YPAD + 0.3M KCl at 30°C for 3 days and removing the biomass by centrifugation, 2 mL of heparin-Sepharose were added, and the mixture was shaken overnight at 4°C, allowing the FGF to adsorb to the heparin-Sepharose. The heparin-Sepharose was subsequently recovered, packed in a column, PBS + 0.01% CHAPS and PBS + 2.5M NaCl + 0.01% CHAPS were used as a solvent, and FGF was eluted from the heparin-Sepharose by increasing the salt concentration.

About 150 μg of purified FGF was applied to a reverse phase column for desalting. The column used was a μRPC C2/C18 PC 3.2/3 column (Pharmacia), and elution from the reverse phase column was carried out using 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid-60% acetonitrile as a solvent.

The sample eluted from the column was dried, and the hydrazine decomposition reaction was carried out. Specifically, 2 mL of hydrazine were added under vacuum and the mixture was treated at 110°C for 60 min. Subsequently, the reaction mixture was cooled to room temperature, and N-acetylation was carried out. The reaction was carried out by adding 250 μL of 0.2M ammonium acetate and 25 μL of acetic anhydride, the mixture was thoroughly stirred and allowed to stand at room temperature for 30 min. Furthermore, 250 μL of 0.2M ammonium acetate and 25 μL of acetic anhydride were added, the mixture was thoroughly stirred and allowed to stand at room temperature for 30 min. The reaction mixture was concentrated to dryness to obtain a saccharide chain preparation.

The following procedures were used to carry out fluorescent-labeling (pyridylamination) of the saccharide chains prepared. Specifically, 20 μL of a coupling reagent (300 mg of 2-aminopyridine dissolved in 100 μL of acetic acid) were added to the saccharide chain preparation concentrated to dryness, the mixture was sealed in a container and treated at 90°C for 60 min. Subsequently, 20 μL of a reducing reagent (10 mg of a borane-dimethylamine complex dissolved in 50 μL of acetic acid) were added, the mixture was sealed in a container and treated at 80°C for 60 min. After completing the reaction, 20 μL of triethylamine-methanol were added, the mixture was thoroughly stirred, 40 μL of toluene were added, the mixture was thoroughly stirred, and subsequently the mixture was concentrated to dryness at 60°C under a flow of nitrogen gas over 10 min. Subsequently, 20 μL of methanol were added, the mixture was thoroughly stirred, 40 μL of toluene was added, the mixture was thoroughly stirred, and subsequently the mixture was concentrated to dryness at 60°C under a flow of nitrogen gas over 10 min. After the procedures were repeated three times, 50 μL of toluene was added to the residue, and the mixture was concentrated to dryness at 60°C under a flow of nitrogen gas by taking 10 min. After completing the reaction, the reaction mixture was treated in an HW-40 gel filtration column to remove any 2 minopyridine not consumed in the reaction.

The saccharide chain structure was analyzed by carrying out HPLC using an amine column. The column used was an Asahipak NH2P-50 (4.6 mm x 250 mm), and a 7:3 mixture (solvent A) of 200 mM acetic acid-triethylamine buffer solution (pH7.3) and acetonitrile and 2:8 mixture (solvent B) of 200 mM acetic acid-triethylamine buffer solution (pH7.3) and acetonitrile were used as a solvent.

The column was equilibrated by allowing solvent A to flow at a flow rate of 1.0 mL/min, immediately after sample injection the proportion of solvent B was increased linearly to 100% over 50 min, and subsequently 100% solvent B was allowed to flow for 20 min to allow the PA-oligosaccharide to elute. Figure 14 shows the analytical results. The TIY48 strain-origin sample showed a single main peak similarly to the results obtained in Application Example 2 (Figure 14, upper), and the elution position was found to coincide with that of the $Man_8GlcNAc_2$-PA standard product (Takara Shuzo). On the other hand, the α-1,2-mannosidase gene-containing TIY53 strain-origin sample showed one main peak (Figure 14, lower). The elution position of the peak was found to coincide with that of the $Man_5GlcNAc_2$-PA standard product. Therefore, the human glycoprotein FGF expressed in the TIY53 strain was found to have roughly 100% of a $Man_5GlcNAc_2$ saccharide chain which is a hybrid or mixed precursor.

Furthermore, an HA-tag-containing gene fragment was excised from the human hepatic α-mannosidase II expression vector pYEOM2-HA using SacI and SphI, and the ends were blunted with T4 DNA polymerase. The fragment was inserted at the SmaI site of pAUR123. After confirming that the promoter was ligated in the correct direction, the α-mannosidase II gene region, including the promoter, was excised with BamHI and inserted at the BamHI site of pRS406. The plasmid prepared was made into a straight chain using NdeI, and the TIY60 strain was transformed using the lithium acetate method. After transformation, it was sown on a plate containing 0.3 M KCl in SD-Ura culture medium [2% glucose, 0.67% yeast nitrogen base w/o amino acids (Difco), nucleic acid bases and amino acid mixture excluding uracil (20-400 mg/L)], and cultured at 30°C for 2 days to obtain a transformant. The genomic DNA of the transformant was prepared, the PCR method was carried out to confirm that the gene was incorporated at the URA3 region of the chromosome, and the strain prepared was named MSY1. The enzymatic activity was measured using the cell extract of the MSY1 strain, confirming the expression of α-mannosidase II.

Application possibility in the industrial field

The auxotrophic triple and quadruple variants of this invention enable the production of high purity neutral saccharide chains in large quantity identical to those high mannose saccharide chains produced by mammals such as humans and glycoproteins having the same neutral saccharide chains. Furthermore, by inserting the genes of the mammalian saccharide chain biosynthesis system into the variants, the efficient production of high mannose, hybrid and complex mammalian saccharide chains and glycoproteins containing mammalian saccharide chains is possible.

Claims

1. A yeast variant having a glycoprotein-producing ability characterized by having the phenotype of the och1, mnn1 and mnn4 mutations and at least 4 kinds or more of auxotrophic phenotypes as well as ability to produce a glycoprotein containing an oligosaccharide chain represented by the following formula (I) as an asparagine-binding saccharide chain.

//Insert top p. 47 the formula (I).//

(In the formula, Man denotes mannose, and GlcNAc denotes N-acetylglucosamine. * denotes a possible phosphorylation site.)

2. A yeast variant having a glycoprotein-producing ability characterized by having the phenotype of the och1 mutation with the OCH1 gene destroyed (Δoch1), the mnn1 mutation with the MNN1 gene destroyed (Δmnn1) and the mnn4 mutation with the MNN41 gene destroyed (Δmnn4) and at least 1 kind or more of auxotrophic phenotypes as well as ability to produce a glycoprotein containing an oligosaccharide chain represented by the following formula (I) as an asparagine-binding saccharide chain.

//Insert the formula (I).//

(In the formula, Man denotes mannose, and GlcNAc denotes N-acetylglucosamine. * denotes a possible phosphorylation site.)

3. The yeast variant of Claim 1 or 2, wherein the auxotrophic phenotypes are selected from the ura3, his3, leu2, ade2, trp1 and can1 mutations.

4. The yeast variant of Claim 3, wherein the yeast belongs to the family *Saccharomyces*.

5. The yeast variant of Claim 4, wherein the yeast belongs to *Saccharomyces cerevisae*.

6. The yeast variant of Claim 5, wherein the yeast is *Saccharomyces cerevisae* TIY19.

7. A process for the production of an oligosaccharide chain characterized by culturing one of the yeast variants of Claims 1-6 in a culture medium, allowing a glycoprotein containing

an oligosaccharide chain represented by the following formula (I) as an asparagine-binding saccharide chain;

//Insert middle p. 48, the formula (I).//

(where Man denotes mannose, and GlcNAc denotes N-acetylglucosamine. * denotes a possible phosphorylation site) to form and accumulate in the culture, collecting the glycoprotein from the culture and recovering the oligosaccharide from the glycoprotein collected.

8. A process for the production of a glycoprotein characterized by culturing one of the yeast variants of Claims 1-6 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain represented by the following formula (I) as an asparagine-binding saccharide chain;

//Insert bottom p. 48, the formula (I).//

(where Man denotes mannose, and GlcNAc denotes N-acetylglucosamine. * denotes a possible phosphorylation site) to form and accumulate in the culture and collecting the glycoprotein from the culture.

9. A process for the production of a glycoprotein characterized by culturing one of the yeast variants of Claims 1-6 transformed by a recombinant plasmid containing the gene coding for a mammalian-origin asparagine-binding glycoprotein in a culture medium, allowing the glycoprotein containing an oligosaccharide chain represented by the following formula (I) as an asparagine-binding saccharide chain;

//Insert p. 49, the formula (I).//

(where Man denotes mannose, and GlcNAc denotes N-acetylglucosamine. * denotes a possible phosphorylation site) to form and accumulate in the culture and collecting the glycoprotein from the culture.

10. A yeast variant characterized by inserting at least two or more mammalian saccharide chain biosynthesis genes into a yeast variant having the och1, mnn1 and mnn4 phenotypes.

11. A yeast variant characterized by inserting at least one or more mammalian saccharide chain biosynthesis genes into a yeast variant of Claims 1-6.

12. A process for the production of an oligosaccharide chain characterized by culturing the yeast variants of Claim 10 or 11 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture, collecting the glycoprotein from the culture and recovering the oligosaccharide from the glycoprotein collected.

13. A process for the production of a glycoprotein characterized by culturing the yeast variants of Claim 10 or 11 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture and collecting the glycoprotein from the culture.

14. A process for the production of a glycoprotein characterized by culturing the yeast variants of Claim 10 or 11 transformed with a recombinant plasmid containing the gene coding for a mammalian-origin asparagine-binding glycoprotein in a culture medium, allowing the glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture and collecting the glycoprotein from the culture.

15. A yeast variant having a glycoprotein-producing ability characterized by having the phenotype of the och1, mnn1, mnn4 and alg3 mutations and at least 5 kinds or more of auxotrophic phenotypes as well as ability to produce a glycoprotein containing an oligosaccharide chain represented by the following formula (II) as an asparagine-binding saccharide chain.

//Insert the formula (II), p. 50//

(In the formula, Man denotes mannose, and GlcNAc denotes N-acetylglucosamine.)

16. A yeast variant having a glycoprotein-producing ability characterized by having the phenotype of the och1 mutation with the OCH1 gene destroyed (Δoch1), the mnn1 mutation with the MNN1 gene destroyed (Δmnn1), the mnn4 mutation with the MNN41 gene destroyed (Δmnn4) and the alg3 mutation with the ALG3 gene destroyed (Δalg3) and at least 1 kind or more of auxotrophic phenotypes as well as ability to produce a glycoprotein containing an oligosaccharide chain represented by the following formula (II) as an asparagine-binding saccharide chain.

//Insert the top p. 51, formula (II).//

(In the formula, Man denotes mannose, and GlcNAc denotes N-acetylglucosamine.)

17. The yeast variant of Claim 15 or 16, wherein the auxotrophic phenotypes are selected from the ura3, the his3, leu2, ade2, trp1 and can1 mutations.

18. The yeast variant of Claim 17, wherein the yeast belongs to the family *Saccharomyces*.

19. The yeast variant of Claim 18, wherein the yeast belongs to *Saccharomyces cerevisae*.

20. The yeast variant of Claim 19, wherein the yeast is *Saccharomyces cerevisae* YS134-4A.

21. A process for the production of an oligosaccharide chain characterized by culturing one of the yeast variants of Claims 15-20 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain represented by the following formula (II) as an asparagine-binding saccharide chain;

//Insert the formula (II).//

(where Man denotes mannose, and GlcNAc denotes N-acetylglucosamine) to form and accumulate in the culture, collecting the glycoprotein from the culture and recovering the oligosaccharide from the glycoprotein collected.

22. A process for the production of a glycoprotein characterized by culturing one of the yeast variants of Claims 15-20 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain represented by the following formula (II) as an asparagine-binding saccharide chain;

//Insert the formula (II), to p. 52//

(where Man denotes mannose, and GlcNAc denotes N-acetylglucosamine) to form and accumulate in the culture and collecting the glycoprotein from the culture.

23. A process for the production of a glycoprotein characterized by culturing one of the yeast variants of Claims 15-20 transformed by a recombinant plasmid containing the gene coding for a mammalian-origin asparagine-binding glycoprotein in a culture medium, allowing the glycoprotein containing an oligosaccharide chain represented by the following formula (II) as an asparagine-binding saccharide chain;

//Insert middle, p. 52, the formula (II).//

(where Man denotes mannose, and GlcNAc denotes N-acetylglucosamine) to form and accumulate in the culture and collecting the glycoprotein from the culture.

24. A yeast variant characterized by inserting at least two or more mammalian saccharide chain biosynthesis genes into a yeast variant having the och1, mnn1, mnn4 and alg3 phenotypes.

25. A yeast variant characterized by inserting at least one or more of mammalian saccharide chain biosynthesis genes into a yeast variant of Claims 15-20.

26. A process for the production of an oligosaccharide chain characterized by culturing the yeast variants of Claim 24 or 25 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture, collecting the glycoprotein from the culture and recovering the oligosaccharide from the glycoprotein collected.

27. A process for the production of a glycoprotein characterized by culturing the yeast variants of Claim 24 or 25 in a culture medium, allowing a glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture and collecting the glycoprotein from the culture.

28. A process for the production of a glycoprotein characterized by culturing the yeast variants of Claim 24 or 25 transformed with a recombinant plasmid containing the gene coding for a mammalian-origin asparagine-binding glycoprotein in a culture medium, allowing the glycoprotein containing an oligosaccharide chain as an asparagine-binding saccharide to form and accumulate in the culture and collecting the glycoprotein from the culture.

29. A yeast strain having α-mannosidase II activity characterized by having the α-mannosidase II gene inserted.

30. A process for the production of α-mannosidase II characterized by culturing the yeast strain of Claim 29 in a culture medium and collecting the α-mannosidase II formed and accumulated in the culture.